Evidence toward a Dual Phosphatase Mechanism That Restricts Aurora A (Thr-295) Phosphorylation during the Early Embryonic Cell Cycle*5

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Background: Aurora A is a spindle-regulating mitotic kinase that has been implicated in controlling CDK1 activation.

Results: Inducing an abnormal increase in Aurora A (Thr-295) phosphorylation during late interphase delays CDK1 activation.

Conclusion: Aurora A (Thr-295) phosphorylation during late interphase competes for phosphatase activity that also promotes CDK1 activation.

Significance: Learning how kinases and phosphatases function as a system is essential to understand cell cycle control.

The mitotic kinase Aurora A (AurA) is regulated by a complex network of factors that includes co-activator binding, autophosphorylation, and dephosphorylation. Dephosphorylation of AurA by PP2A (human, Ser-51; Xenopus, Ser-53) destabilizes the protein, whereas mitotic dephosphorylation of its T-loop (human, Thr-288; Xenopus, Thr-295) by PP6 represses AurA activity. However, AurA(Thr-295) phosphorylation is restricted throughout the early embryonic cell cycle, not just during M-phase, and how Thr-295 is kept dephosphorylated during interphase and whether or not this mechanism impacts the cell cycle oscillator were unknown. Titration of okadaic acid (OA) or fotsricin into Xenopus early embryonic extract revealed that phosphatase activity other than PP1 continuously suppresses AurA (Thr-295) phosphorylation during the early embryonic cell cycle. Unexpectedly, we observed that inhibiting a phosphatase activity highly sensitive to OA caused an abnormal increase in AurA (Thr-295) phosphorylation late during interphase that corresponded with delayed cyclin-dependent kinase 1 (CDK1) activation. AurA (Thr-295) phosphorylation indeed influenced this timing, because AurA isoforms retaining an intact Thr-295 residue further delayed M-phase entry. Using mathematical modeling, we determined that one phosphatase would be insufficient to restrict AurA phosphorylation and regulate CDK1 activation, whereas a dual phosphatase topology best recapitulated our experimental observations. We propose that two phosphatases target Thr-295 of AurA to prevent premature AurA activation during interphase and that phosphorylated AurA (Thr-295) acts as a competitor substrate with a CDK1-activating phosphatase in late interphase. These results suggest a novel relationship between AurA and protein phosphatases during progression throughout the early embryonic cell cycle and shed new light on potential defects caused by AurA overexpression.

Protein phosphorylation is a regulatory mechanism pervasive to cell division control. Cyclin-dependent kinase 1 (CDK1), 2 Aurora A (AurA), and Polo-like kinase 1 (Plk1) are mitotic kinases that phosphorylate specific substrates during multiple mitotic events (1, 2). Counterparts to these enzymes are the phosphoprotein phosphatases that dephosphorylate substrates and drive mitotic entry, progression, and exit (3). Mitotic kinases and phosphatases remain topics of intense study, particularly regarding their roles in the processes of bipolar spindle formation, chromosome segregation, spindle checkpoint control, and cytokinesis (3). There is also an emerging interest in understanding how the opposing activities of phosphatases drive these processes (4–6). The present study culminated with our characterizing a differential regulation of AurA (Thr(P)-295) dephosphorylation that occurs during the early embryonic cell cycle and our assessment of its impact on the CDK1 oscillator.

AurA is constantly present in Xenopus early embryos and inactive during interphase, whereas in mammalian cells, it must be newly translated during interphase because it is degraded after it functions in mitosis (7–9). The regulation of AurA activity involves an intricate network linked with protein co-factors, kinases, and phosphatases, and since its discovery, many mechanisms have been proposed for the trigger of AurA activation at the G2-M transition. One proposed model includes AurA dephosphorylation by protein phosphatase 1 (PP1) during interphase in human cells (10). At mitotic entry, the binding of targeting protein for Xklp2 (TPX2), released during nuclear envelope breakdown (NEBD), recruits AurA to the spindle microtubules and promotes AurA T-loop autophosphorylation (human Thr-288/Xenopus Thr-295) (10–14). A second model has the 192-kDa centrosomal protein (Cep192) targeting AurA to the centrosomes, where it forms homodimers or oligomers to become activated (15); additional factors, such as Ajuba, Arpc1b, HEF1, PAK1, and nucleophosmin/B23, also serve to

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2 The abbreviations used are: CDK1, cyclin-dependent kinase 1; AurA, Aurora A; Plk1, Polo-like kinase 1; NEBD, nuclear envelope breakdown; OA, okadaic acid; P-AurA, AurA phosphorylated at its T-loop; MBP, maltose-binding protein; rAurA, recombinant active AurA; dEnsa (S68D), D. melanogaster α-endosulfine S68D; KD, kinase-dead; IP, immunoprecipitation.
activate AurA on centrosomes (16–20). A third model involves a positive feedback loop between Plk1 and AurA (21), which further recruits and activates AurA on centrosomes (22–26). Not only is the proper timing of AurA activation important to cell cycle progression, but its activity must also be strictly controlled. Overexpression of AurA did not accelerate mitosis, but it arrested proliferation in primary and transformed human cells either before or during cell division (27–29). These studies underscored that AurA activity must be limited throughout the cell cycle, including during mitosis. Recent work uncovered an essential AurA T-loop phosphatase in human cells, protein phosphatase 6 (PP6), which restricts AurA activity during M phase (4, 6). Together, these reports raised some intriguing questions. How is AurA phosphorylation restricted before mitotic entry during early embryonic oscillations when AurA is not degraded? Might this regulation play any role in cell cycle timing?

In addition to the functions of mitotic kinases, new phosphoprotein phosphatases have been identified that function in mitosis as well as interphase. Besides PP1 and PP6, the phosphoprotein phosphatase family also includes PP2A, PP2B, PP4, PP5, and PP7. Their catalytic subunits are similar, with all inhibited by the phosphatase inhibitor okadaic acid (OA). However, they vary in their sensitivity to OA due to subtle structural differences (3). This makes OA and other inhibitors useful to distinguish between certain phosphoprotein phosphatase activities in vivo and in vitro, namely PP1 and the other phosphatases that are more sensitive to OA (30). Recent studies have shown that PP2A and PP1 recognize different sets of substrates that direct mitotic entry, progression, and exit (31–35). A specific isoform of PP2A, B55δ, is known to inhibit CDK1 activation and is inhibited by the Greatwall kinase (5, 36). PP2A colocalizes and interacts with AurA during interphase and mitosis (37), dephosphorylating AurA(Thr(P)-295) (human; Xenopus, Ser-53) and promoting its APC-Cdh1-mediated degradation in somatic cells (8, 38). However, AurA destabilization does not occur during the early embryonic cell cycle, and its capacity for autophosphorylation suggests that an unidentified phosphatase holds Thr-295 dephosphorylated prior to mitotic entry. At this juncture, if and how AurA phosphorylation and/or activity are restricted during interphase have not been addressed.

Through careful titration of OA into Xenopus early embryonic extracts, we reveal that phosphorylation of AurA at its T-loop (Thr-295) (P-AurA) is restricted throughout interphase by phosphatase activities other than PP1. We discovered that low OA concentrations caused an abnormal increase in P-AurA levels later during interphase and did not accelerate but rather delayed CDK1 activation. We also found that increasing P-AurA levels by adding recombinant AurA to either intact or AurA-depleted extracts also delayed CDK1 activation. We identified PP2A as a likely candidate for the AurA interphase phosphatase but excluded PP2A-B55δ because its inhibition by phosphomimetic Drosophila Ensa (S68D) protein did not cause P-AurA accumulation. Using mathematical modeling, we determined that the differential activities of two phosphatases, potentially PP2A and PP6, are required to recapitulate the phosphorylation pattern of AurA(Thr-295) and the timing of CDK1 activation observed in our experiments. In summary, our study underscores the importance of restricting AurA phosphorylation during interphase as the early embryonic cell cycle progresses and reveals how integration of both kinase and phosphatase activities can be critical to generate biological responses that are precisely timed and tuned.

**EXPERIMENTAL PROCEDURES**

Constructs, Site-directed Mutagenesis, and Protein Expression—Full-length Xenopus laevis AurA cDNA cloned into vector pET21 was a gift from C. Walczak (Indiana University, Bloomington, IN). Site-directed mutagenesis using PCR was performed to generate kinase-dead (KD) D281A, T294A/T295A, and T295D mutants. Sense (5′-CTGAAAGATCGCGC-TCCTTTGGCTGTC-3′) and antisense primers (5′-TTTCTCC-ATTTGAGCGACGAGCG-3′) were used to mutate aspartic acid 281 (GAC) to alanine (GCC). Sense (5′-TCCAG-GAGGGCCCGCCTGTGTGGAAC-3′) and antisense primers (5′-GGATGGACATGCAGACGAGCC-3′) were used to mutate threonyne 294 (ACC) and threonyne 295 (ACT) to alanine (GCC). Sense (5′-TCCAGGAGGACCGATCTGTGGTA-ACG-3′) and antisense primers (5′-GGATGGACATGCGAGCA-GACGAGGC-3′) were used to mutate threonine 295 (ACC) and threonine 295 (ACT) to aspartic acid (GAT). *Xenopus* Eg5 stalk fragment (EQMDRRRIYISQVR) was cloned into pGEX4T-1 in frame with GST using the following DNA oligonucleotides: forward (5′-GATCCGAACAAATGTGATAGACGCTTCAGTGTGATTCA-GCAGACGAGCC-3′) and reverse (5′-TCCAGTCCGTGACATGCAGACGACGAGCGACGAGCC-3′). *Drosophila melanogaster* α-endsolinlite S68D (dEnsa (S68D)) mutant cloned into vector pMAL-c2x was a gift from M. Goldberg (Cornell University). Non-degradable *Xenopus* cyclin B1 (Δ65XCycB1) was used previously (39). His-tagged proteins, GST fusion proteins, or maltose-binding protein (MBP) fusion proteins were expressed in *Escherichia coli* BL21 (DE3)pLysS that was transformed with corresponding clones, induced with 1 mM isopropyl-1-thio-D-glactopyranoside overnight at 18 °C, and purified using TALON® metal affinity resin (Clontech), glutathione-Sepharose beads (Milenyi Biotech), or amylose resin (New England Biolabs), respectively, according to the manufacturer’s protocols. Eluted proteins were dialyzed into dialysis buffer (150 mM NaCl, 50 mM sucrose, 10 mM Hepes-NaOH (pH 7.7), 2.5 mM MgCl2 (pH 7.5)). Protein purity was determined by SDS-PAGE and Coomassie Blue staining. Protein concentrations were determined by a BCA protein assay kit (Thermo Fisher Scientific), with bovine serum albumin used as the standard.

**Antibodies**—Rabbit polyclonal anti-AurA used for Western blot and immunoprecipitation was generated against a C-terminal peptide (CKNSQLKKDPELPLGAQ) (The first Cys residue in the N terminus is added additionally during peptide synthesis) derived from the sequence of *Xenopus* AurA (40). Antibodies and the concentrations at which they were used are as follows: mouse anti-cyclin B2 (Santa Cruz Biotechnology, Inc., sc-53240) (0.2 μg/ml), rabbit anti-His (Santa Cruz Biotechnology, sc-803) (0.2 μg/ml), mouse anti-MBP (New England Biolabs, E8032) (0.2 μg/ml), rabbit anti-CDK1(Thr(P)-15) (Cell Signaling Technology, 9111) (1:2,000), rabbit anti-AurA(Thr(P)-288) (Cell Signaling, 3079) (1:5,000), rabbit
anti-Cdc25C(Ser(P)-216) (Cell Signaling, 9528) (1:1,000), mouse anti-α-tubulin (Sigma-Aldrich, T6199) (0.1 μg/ml), rabbit anti-β-casein (Ambion International, P046) (1:1000), mouse anti-PP2A-C (Millipore, 05-421) (0.2 μg/ml), mouse anti-PP2A-B56e (Millipore, MABS270) (1 μg/ml), rabbit anti-Wee1 (Invitrogen, 51-1700) (0.5 μg/ml), mouse anti-FLAG (Sigma-Aldrich, F1804) (0.5 μg/ml).

Phospho-AurA(xThr-295/hThr-288) and Phospho-Cdc25C(xSer-287/hSer-216) Immunoblotting—Anti-phospho-human AurA(Thr-288) and anti-phospho-human Cdc25C(Ser-216) antibodies were used previously to detect the corresponding Xenopus proteins in egg extracts because of the conservation of the sequences of the antigens (15, 41). One-quarter microliter of extracts in a 1:50 dilution with EB buffer (80 mM potassium hydroxide, 20 mM EGTA, and 15 mM MgCl2 (pH 7.3)) was electrophoresed through 12.5% Criterion gels (Bio-Rad) and transferred onto PVDF (Millipore). Membranes were blocked with Odyssey blocking buffer (LI-COR) for 1 h at 21 °C, stripped with stripping buffer (25 mM glycine, 1% SDS (pH 2.0)) for 30 min, blocked with 3% milk in TBS buffer (50 mM Tris-Cl (pH 7.5), 150 mM NaCl), incubated with anti-AurA(Thr-P-288) or anti-Cdc25C(Ser-P-216) antibodies in 2% BSA-TBS at 4 °C overnight and with IRDye® secondary antibodies (LI-COR) for 1 h at 21 °C, and analyzed using Odyssey software (LI-COR).

In Vitro Transcription—mRNA was transcribed using a mMessage mMachine SP6 kit (Ambion) with Xenopus AurA and PP2A-B55e, pCS111CFLAGHis templates linearized by Ascl. RNA concentration was determined by spectrophotometry, diluted to 1 mg/ml, visualized by electrophoresis through a non-denaturing 1% Tris acetate-EDTA gel, and stored at −80 °C.

Experiments Using Xenopus Egg Extracts—Cycling extracts and sperm chromatin were prepared as described previously (39), as were interphase extracts (42). Typically, demembranated Xenopus sperm chromatin was added to a concentration of 104/μl extract, unless otherwise specified. All assays in extracts were carried out at 21 °C, unless otherwise specified. Where indicated, 1:100 dilution of OA (LC Laboratories), fos-triecin (Santa Cruz Biotechnology), or DMSO solvent only was added. Cycloheximide was used at a 1:100 dilution at 100 μg/ml (in water) in egg extracts. Roscovitine (Cayman Chemical) was used at a 1:156 dilution at 0.18 mM (in ethanol). MBP-dEnsa (S68D) was used at 10 or 30 μM, and αPP1-C (New England Biolabs) and PP2A-A/C dimer (Millipore, lot number 2044810) were used at 0.012 units/μl. Samples of egg extract were removed at various intervals and frozen on dry ice for subsequent assays. For co-immunoprecipitation of AurA and PP2A-C extracts, 150 μl of cycling extract at 20 min was diluted with 3 volumes of XB buffer (100 mM KCl, 10 mM Hepes-KOH, 0.1 mM CaCl2, 1 mM MgCl2, 50 mM sucrose (pH 7.7)) and incubated with 20 μg of the indicated antibodies coupled to 40 μl of protein G Dynabeads for 2 h at 4 °C. Dynabeads were then either washed twice with XB (“mild”), or twice with XB and twice with XB + 500 mM KCl (“stringent”). Co-precipitating proteins were analyzed by immunoblotting. Immunodepletion of AurA from undiluted extract was also accomplished using the protocol described above.

Cell Cycle Restriction of AurA(Thr-295) Phosphorylation

Kinase and Phosphatase Assays—Histone H1 kinase assays were performed with 5 μl of EB-diluted egg extract samples (1:50 dilution), which were incubated with an equal volume of kinase buffer containing 1 mg/ml histone H1, 400 μM ATP, 20 μM PKA inhibitor (Santa Cruz Biotechnology), 1.25 μCi of [γ-32P]ATP (PerkinElmer Life Sciences), 25 mM Hepes-KOH (pH 7.7), 6 mM EGTA, and 12.7 mM MgCl2. AurA was immunoprecipitated from 2 μl of egg extract samples in 125 μl of immunoprecipitation buffer (XB buffer containing 80 mM β-glycerophosphate) with 1.5 μg of anti-AurA antibody coupled to 8 μl of protein G Dynabeads and transferred onto PVDF (Millipore). Membranes were blocked with Odyssey blocking buffer (LI-COR) for 1 h at 21 °C, stripped with stripping buffer (25 mM glycine, 1% SDS (pH 2.0)) for 30 min, blocked with 3% milk in TBS buffer (50 mM Tris-Cl (pH 7.5), 150 mM NaCl), incubated with anti-AurA(Thr-P-288) or anti-Cdc25C(Ser-P-216) antibodies in 2% BSA-TBS at 4 °C overnight and with IRDye® secondary antibodies (LI-COR) for 1 h at 21 °C, and analyzed using Odyssey software (LI-COR).

RESULTS

Phosphatase Activity Suppresses AurA Phosphorylation during Interphase in Xenopus Early Embryonic Extract—An earlier study investigating AurA activation at centrosomes revealed that Thr-295 phosphorylation of AurA (P-AurA), commonly used as a biomarker for AurA activity, was undetectable in Xenopus meiotic metaphase II-arrested cytosolic factor (CSF) extract that either lacked or had a small number of added centrosomes (15). Sperm nuclei are a source of functional centrosomes (44), and cycling egg extract containing a low concentration of sperm nuclei (103/μl) produced negligible changes in P-AurA during the cell cycle (Fig. 1A). Direct measurement of CDK1 and AurA activities in cycling extract revealed that the latter increased roughly 2-fold during mitosis and peaked after CDK1 (Fig. 1B). These observations prompted a hypothesis, that the lack of AurA phosphorylation during early interphase occurs due to sustained phosphatase activity. To test this, we measured the effects of sperm addition and/or phosphatase inhibition on AurA phosphorylation in egg extract. Adding a high concentration of sperm nuclei to egg extract (4 × 104/μl) had been shown previously to cause P-AurA accumulation (15), and our results agreed (Fig. 1C, compare left two panels). However, adding phosphatase inhibitor (1 μM OA) in the absence of sperm more than doubled the level of AurA phosphorylation.
induced by sperm addition, whereas also adding sperm did not further increase P-AurA accumulation (Fig. 1C, compare right two panels). Therefore, although centrosomal factors do stimulate AurA phosphorylation, this phosphorylation is being continually suppressed by phosphatase activity at the start of the early embryonic cell cycle.

**Phosphatases Other than PP1 Dephosphorylate AurA(Thr-295) during Early Embryonic Interphase**—Our data above suggested that phosphatase activity holds AurA dephosphorylated during interphase of the early embryonic cell cycle. A prior cell cycle study using HeLa cells implicated PP1 as an interphase AurA phosphatase (10). PP1 is a plausible candidate to dephosphorylate AurA during interphase in early embryonic extracts as well because it is known to also regulate other cell cycle targets, including Cdc25C. Phosphorylation of Cdc25C at Ser-287 (Cdc25(p287)) (Xenopus; equivalent to Ser-216 of human Cdc25C) (45) during interphase corresponds with its inactive state. Its dephosphorylation by PP1 at Ser-287 activates Cdc25C at mitotic entry, whereas phosphorylation at other sites decreases its electrophoretic mobility (45, 46).

Using the differential sensitivity of the phosphatases PP1 and PP2A (and others) to OA, we titrated decreasing concentrations into cycling egg extract to determine if PP1 dephosphorylation of Cdc25C(p287) would correspond with AurA(Thr(P)-295) dephosphorylation. Prior work reported that PP1 activity is reduced by ∼50% in egg extract treated with 900 nM OA (47), so we tested 1 μM OA and lower concentrations (Fig. 2). In contrast to DMSO-treated extract, 1 μM OA prevented a total phospho-shift of Cdc25C and complete loss of Cdc25C(Ser(P)-287) signal before CDK1 activity reached its maximum (between 20 and 30 min) (Fig. 2, A (compare black squares with black circles) and B). This suggested that PP1 was still partially inhibited in the presence of 1 μM OA. However, 400 nM OA did not inhibit PP1, as seen by the complete loss of Cdc25C(Ser(P)-287) at mitosis, but P-AurA and AurA activity increased substantially within 30 min, concomitant with early CDK1 activation (Fig. 2, A (compare gray diamonds and black circles) and B). The observation that 400 nM OA accelerated CDK1 activation underscored earlier findings that the half-maximal concentration (IC_{50}) of OA to inhibit PP2A-B55δ, a known inhibitor of mitotic entry in egg extract, is 200–300 nm (33). Surprisingly, the 100 nM treatment with OA did not cause early CDK1 activation and delayed it instead (Fig. 2, A (compare black squares and black dots) and B). This unexpected delay in mitotic entry caused by 100 nM OA suggested the inhibition of a phosphatase activity, separate from the CDK1-inhibiting PP2A-B55δ, that promotes rather than inhibits CDK1 activation during late interphase and just prior to mitotic entry. Because OA is a broad spectrum phosphatase inhibitor, we tested the effect of the PP2A-specific inhibitor fostriecin on AurA(Thr(P)-295) phosphatase and CDK1 activation (48, 49). Titration of this inhibitor into cycling egg extract to concentrations from 1 to 10 μM also increased AurA(Thr(P)-295) in conjunction with an accelerated CDK1 activation (Fig. 2C).

Together, these data exclude PP1 as the P-AurA(Thr(P)-295) phosphatase early in interphase of the embryonic cell cycle.
This implicated that at least two types of phosphatase activity operate during that time: first, the early activity that represses CDK1 activation, namely PP2A-B55δ/H9254, which may also hold AurA(Thr-295) dephosphorylated at the start of the cycle, and second, an activity that is more sensitive to OA, which influences CDK1 activation near the end of interphase.

Phosphatase Inhibition Permits Aurora A Activation Independent of CDK1 Activity—Phosphatase activity restricts the activating phosphorylation of AurA during the embryonic cell cycle, and we found that its peak activity follows that of CDK1 (Fig. 1B). Views have been mixed regarding the timing of activation and the dependences of these kinases on each other during an embryonic cycle. Some groups have reported that CDK1 is downstream of AurA (26, 50), whereas others concluded that AurA activation first requires CDK1 activity (51, 52). Our discovery that either OA or fostriecin treatment causes an abnormal increase in P-AurA during early interphase led us to test the hypothesis that suppressing phosphatase activity would permit AurA activation in the absence of CDK1 activity. Treating cycling egg extract with 20 \( \mu M \) OA, a concentration reported to inhibit PP1 and other phosphoprotein phosphatases (35), rapidly increased CDK1 activity ~5-fold within 30 min (Fig. 3A).
(left, black dots)). This agreed with prior reports that inhibiting PP2A-B55δ by OA accelerated and blunted CDK1 activation, even with low cyclin and Cdc25 activity levels (31, 33, 47). This also strongly increased P-AurA levels and AurA activity (~13-fold) relative to DMSO and EtOH (Fig. 3, A (right, compare black squares and black dots) and B (top and middle panels)). However, activation of AurA could occur independently of a CDK1 oscillation, when cycling egg extracts were treated with 20 μM OA plus the CDK1 inhibitor, roscovitine (Fig. 3, A (gray circles) and B (bottom panel)). Here, roscovitine and OA treatment together permitted a significant increase in neither cyclin B2 nor CDK1 activity relative to their vehicles, but AurA(Thr-295) was still strongly phosphorylated. We also tested the effects of sperm and/or OA addition on AurA phosphorylation in interphase extract, where cyclin synthesis is blocked by cycloheximide and CDK1 remains inactive. In cycloheximide-supplemented interphase extract with and without added sperm chromatin, P-AurA appeared in the 400 nM OA treatment but not the DMSO controls (Fig. 3C, compare left and right panels). Therefore, in interphase egg extract with phosphatases suppressed by as little as 400 nM OA, neither CDK1 activity nor centrosomes are required for AurA activation. In conclusion, it is continually operating phosphatase activity independent of PP1, possibly an isoform of PP2A (e.g. PP2A-B55δ) and/or other phosphatases, such as PP6, that restricts an increase in AurA phosphorylation and activity throughout interphase progression in Xenopus early embryonic extracts.

Adding Low OA Doses to Cycling Egg Extract Delays Mitotic Entry—We showed that increased AurA activity coincides with a premature and blunted CDK1 activation when cycling extracts are treated with ≥400 nM OA and that CDK1 activity is not necessary for AurA(Thr-295) phosphorylation (Figs. 2A and 3). However, it was unknown whether the same phosphatase activity responsible for suppressing CDK1 activation, namely PP2A-B55δ (33), also holds AurA(Thr-295) dephosphorylated during interphase, so we set out to determine if AurA phosphorylation/activation and the steps that lead to CDK1 activation respond differently to decreasing concentrations of OA. Decreasing OA doses were added to cycling egg extract, and after 30 min, while extracts were still in early interphase (S), AurA(Thr-295) phosphorylation/activity was measured. Indeed, 140–110 nM OA activated AurA within 30 min in cycling extract (Fig. 4, A and B). AurA phosphorylation preceeded CDK1 activation, which was delayed relative to the DMSO control in treatments ranging from 140 to 100 nM OA (Fig. 4C). We also found that the appearance of P-AurA in the 120 nM OA-treated extract corresponded with continued cyclin B2 accumulation and delayed CDK1 activation, relative to the 400 nM OA treatment (Fig. 4D). Thus, later increases in AurA phosphorylation at such low concentrations of OA suggested that a highly OA-sensitive phosphate activity later in interphase has an impact on the firing of CDK1 activation.

We hypothesized that the lack of AurA activation by 30 min in 100 nM OA-treated extract occurred due to an increase the lag in time prior to P-AurA accumulation compared with higher concentrations of inhibitor while also diminishing its abundance; less P-AurA was observed in treatments of OA ranging from 140 to 110 nM (Fig. 4, A and B). To test this hypothesis, we assayed in a time-independent fashion which concentrations of OA would promote P-AurA accumulation in extracts arrested in mitosis. Indeed, as low as 60 nM increased AurA phosphorylation and activity (Fig. 4, E and F). This demonstrated that AurA Thr-295 phosphorylation does occur at 100 nM OA and could be a contributing factor to the delay in mitotic entry at that concentration. One explanation for the lack of P-AurA accumulation at 100 nM OA during a CDK1 oscillation could be that P-AurA is a preferred substrate over
FIGURE 4. Low concentrations of OA increase AurA(Thr-295) phosphorylation and correspond with delayed mitotic entry in early embryonic extract. A, immunoblots and phosphorimages of AurA(Thr(P)-295), AurA, and H1 kinase activities, respectively, from cycling extract with increasing OA doses and sperm chromatin (10^3/µl). Aliquots were withdrawn at 30 min after adding OA into cycling extracts at the indicated concentrations and then immunoblotted. B, histograms of relative AurA(Thr(P)-295) signals (normalized to total AurA signal) and IP AurA activities shown in A. Data are plotted as means ± S.E. (error bars; n = 3). *, p < 0.01 compared with the 0 nM OA treatment, as determined by one-way analysis of variance. C, time courses of H1 kinase activities of cycling egg extracts titrated with increasing concentrations of OA and containing sperm chromatin (10^3/µl) (representative of duplicate experiments). D, immunoblots from cycling extracts with 120 nM (top) or 400 nM OA (bottom) shown in C. The band appearing in the first lane of the cyclin B2 immunoblot (indicated by the dot) in the left panel is the 50-kDa protein marker. Bars, time points where NEBD was observed. Arrows, probed target; asterisks, nonspecific cross-reacting band. E, immunoblots and phosphorimages of AurA(Thr(P)-295), AurA, and H1 kinase activities, respectively, within a series of increasing OA doses in mitosis-arrested extracts containing sperm chromatin (10^3/µl). Non-degradable cyclin B1 (88 nM) was used to induce mitotic arrest. Extract aliquots were withdrawn at 30 min after the addition of OA into mitosis-arrested extracts at the indicated concentrations and immunoblotted. F, quantitated graphs of relative AurA(Thr(P)-295) signals (normalized to total AurA signal) and immunoprecipitated AurA activities shown in E. Quantitated data are plotted as means ± S.E. (error bars; n = 3). *, p < 0.01 compared with the 0 nM OA treatment, as determined by one-way analysis of variance.
the hypothetical CDK1-activating phosphatase activity. This would mean that when a concentration of OA is added that is sufficient to impede phosphatase function, the first phenotype observed would be a delay in CDK1 activation, with little or no initial accumulation of P-AurA. Together, these data uncovered a correlation between an increase in AurA phosphorylation later during interphase with a delay in CDK1 activation.

**Increasing the AurA Concentration in Cycling Egg Extract Delays Mitotic Entry**—AurA has been implicated in many studies to promote mitotic entry, and our current work reveals that increasing the AurA concentration in cycling extract delays mitotic entry.

**FIGURE 5. Increasing the AurA concentration in cycling extract delays mitotic entry.** A, plot of H1 kinase activities in cycling extract treated with 1, 2, or 3 μM His-tagged recombinant AurA, along with [35S]methionine; 3 μM GST protein is included as a negative control. All extracts contained sperm chromatin (103/μl). Aliquots were taken at the indicated time points for H1 kinase assays and 35S-labeled cyclin B precipitation. Representative results of duplicate experiments are shown. The concentration of endogenous AurA in egg extract is 11011M. B, H1 kinase activities and protein levels shown in A; immunoblotting was performed with the indicated antibodies. C, quantitative plots of GST- and His-tagged AurA-treated cycling extracts relative to the buffer-treated extracts, presenting the delay in peak CDK1 activation (min), the gain in maximum cyclin B produced (%), and residual cyclin B remaining at mitotic exit (%). Quantitated data are plotted as means ± S.E. (error bars; n = 2). D, plot of H1 kinase activities in cycling extract treated with 120 nM OA or 3 μM recombinant AurA. Data are plotted as means ± S.E. (error bars; n = 3); E, immunoblots of protein levels in D, probed with the indicated antibodies. Bars in all instances denote incidence of NEBD.
Cell Cycle Restriction of AurA(Thr-295) Phosphorylation

AurA activation can be forced to occur independently of CDK1. This was apparent when AurA was activated 30–40 min into the cell cycle in the presence of low concentrations of OA, a condition that also delayed CDK1 activation (Fig. 4, A–D). It was unclear whether this early gain in AurA activity and delay in CDK1 activation were coincidental or somehow functionally linked, but it raised the intriguing hypothesis that P-AurA(Thr-295) is targeted by a phosphatase activity that also promotes CDK1 activation during late interphase. To test this hypothesis, we first titrated recombinant active AurA (rAurA) into cycling egg extract and measured its effect on a CDK1 oscillation. The endogenous AurA concentration in egg extract is estimated to be 40 ng/µL (~1 µM) (53), so to remain within physiological levels, we tested a range of 1–3 µM rAurA. One micromolar rAurA (already autophosphorylated upon purification) was quickly dephosphorylated in cycling extract and did not delay CDK1 activation, with phospho-Tyr-15-CDK1 signal and cyclin B abundance matching those of buffer- and GST-treated control extracts (Fig. 5, A–C (compare black squares, black dots, and gray diamonds)). However, the addition of either 2 or 3 µM rAurA caused an abnormal increase in P-AurA and did not accelerate CDK1 activation but rather delayed it by an average of 15 and 18 min, respectively (Fig. 5, A and B). This delay corresponded with a prolonged period of cyclin B accumulation followed by its incomplete degradation (Fig. 5C).

Low concentrations of OA induced an increase in P-AurA levels later in interphase and prior to mitosis that corresponded with a delay in CDK1 activation, and we next wanted to determine if this effect was specific to the reduction of a P-AurA-phosphatase activity during that period. To test this, we directly compared cycling egg extracts treated with either 3 µM rAurA or 120 nM OA, the dose that had generated increased P-AurA prior to mitosis (Fig. 5, D and E). Treatment with 120 nM OA delayed mitosis and corresponded with an early and abnormal increase in P-AurA just prior to CDK1 activation (Fig. 5, D (black dots) and E). Conversely, despite an immediate increase in P-AurA signal, rAurA treatment did not accelerate CDK1 activation (as seen in extracts treated with doses of OA ≥400 nM) (Fig. 5, D (gray diamonds) and E). Instead, CDK1 activation was further delayed, and mitotic arrest occurred in correspondence with elevated levels of cyclin B2 (Fig. 5E). Altogether, these data provide evidence that increased levels of P-AurA that are present during late interphase, whether by supplementing recombinant AurA protein or low concentrations of OA into an early embryonic extract, delay mitotic entry. These phenotypes required the presence of rAurA protein at the start of the cycle. The addition of AurA during late interphase in cycling extract as described in a prior study (50) did not accelerate mitosis in our experiments, but it did not cause the delay in CDK1 activation (Fig. 6A). Treatment of cycling extract with rAurA mRNA also delayed CDK1 activation and caused continued cyclin B synthesis, relative to a luciferase (luc) mRNA-treated control (Fig. 6, B–D). These results excluded any potential artifacts caused by the rAurA protein preparation.

Adding Inactive AurA with a Preserved Thr-295 Residue to Cycling Extract Delays Mitotic Entry—Supplementing rAurA into cycling egg extract delayed mitosis, but it was unclear whether this was caused by AurA phosphorylating itself and/or
FIGURE 7. Adding inactive AurA with intact Thr-295 to cycling egg extract delays mitotic entry. A, kinase assays of recombinant wild-type or inactive (kinase-dead) His-tagged AurA mutants using GST-Eg5 peptide as substrate. B, immunoblots of AurA(Thr(P)-295) (top) and total AurA (bottom) from cycling extract treated with His-tagged AurA KD (D281A) and/or 400 nM OA, plus sperm chromatin (103/H9262l). The arrows indicate probed target; asterisks indicate nonspecific cross-reacting band. C, plot of H1 kinase activities in cycling extract treated with 3 M recombinant His-tagged AurA WT or various His-tagged AurA kinase-dead mutants and sperm chromatin (103/μl). Data are plotted as means ± S.E. (error bars; n = 2). D, protein levels from time course in C; immunoblotting was performed with the indicated antibodies (note that exposure for AurA(Thr(P)-295) immunoblot in 3 M AurA WT-treated sample was reduced relative to others). The band appearing in the first lane of the cyclin B2 immunoblot (indicated by the dot) is the 50-kDa protein marker. Bars, time points where NEBD was observed. E, AurA and α-tubulin immunoblots of AurA-depleted Xenopus cycling egg extract supplemented with buffer or various His-tagged AurA proteins (top) and H1 kinase activities in these extracts during a time course (bottom), representative of triplicate experiments. NEBD for each treatment is indicated above the plot, and the average delay (min) in NEBD relative to buffer-treated AurA-depleted extract is shown in the inset (± S.E. (error bars); n = 3).
Cell Cycle Restriction of AurA(Thr-295) Phosphorylation

A, dephosphorylation of AurA by recombinant PP1α-C and PP2A-A/C dimer. Recombinant His-tagged AurA was incubated with either 0.25 units of PP1α-C (New England Biolabs) or PP2A-A/C dimer at 30 °C for 55 min. Reactions were stopped at 0 and 55 min, subjected to SDS-PAGE, and then immunoblotted with anti-AurA (phospho-Thr-288) and anti-His antibodies. Remaining phospho-AurA(Thr-295) signals were quantitated by LI-COR Odyssey software, normalized to the total AurA (via His tag) signals, and a ratio was generated by comparison with the 0-min AurA(Thr(P)-295)/AurA signal. Phosphatase activity is plotted as means ± S.E. (error bars; n = 2). *, p < 0.01 between the two phosphatase treatments, as determined by one-way analysis of variance. B, immunoblots of AurA(Thr(P)-295) in cycling extracts treated with OA in the absence or presence of recombinant PP2A-A/C dimer plus sperm chromatin (10^7/μl). Extract aliquots were withdrawn 90 min following the addition of OA and PP2A, and samples were immunoblotted for the indicated proteins. C, immunoblots of AurA activation in cycling extracts with or without OA in the absence or presence of recombinant PP1. D, immunoblots of immunoprecipitated AurA and catalytic subunit of PP2A from cycling extract during S phase under mild (top) or stringent (bottom) wash conditions. Mock IgG, AurA, and PP2A catalytic subunits precipitated (PP2A-C) precipitations were performed using egg extract containing sperm chromatin (10^7/μl) that was permitted to cycle for 20 min (early interphase). IPs were washed with either XB buffer (mild) or 500 mM KCl-XB buffer (stringent) and then immunoblotted. The first lane from the left was loaded with 1% of extract input. Prolonged exposures of PP2A-C and AurA immunoblots are indicated (long).

FIGURE 8. PP2A associates with AurA in Xenopus early embryonic extract. A, dephosphorylation of AurA by recombinant PP1α-C and PP2A-A/C dimer. Recombinant His-tagged AurA was incubated with either 0.25 units of PP1α-C (New England Biolabs) or PP2A-A/C dimer at 30 °C for 55 min. Reactions were stopped at 0 and 55 min, subjected to SDS-PAGE, and then immunoblotted with anti-AurA (phospho-Thr-288) and anti-His antibodies. Remaining phospho-AurA(Thr-295) signals were quantitated by LI-COR Odyssey software, normalized to the total AurA (via His tag) signals, and a ratio was generated by comparison with the 0-min AurA(Thr(P)-295)/AurA signal. Phosphatase activity is plotted as means ± S.E. (error bars; n = 2). *, p < 0.01 between the two phosphatase treatments, as determined by one-way analysis of variance. B, immunoblots of AurA(Thr(P)-295) in cycling extracts treated with OA in the absence or presence of recombinant PP2A-A/C dimer plus sperm chromatin (10^7/μl). Extract aliquots were withdrawn 90 min following the addition of OA and PP2A, and samples were immunoblotted for the indicated proteins. C, immunoblots of AurA activation in cycling extracts with or without OA in the absence or presence of recombinant PP1. D, immunoblots of immunoprecipitated AurA and catalytic subunit of PP2A from cycling extract during S phase under mild (top) or stringent (bottom) wash conditions. Mock IgG, AurA, and PP2A catalytic subunits (PP2A-C) precipitations were performed using egg extract containing sperm chromatin (10^7/μl) that was permitted to cycle for 20 min (early interphase). IPs were washed with either XB buffer (mild) or 500 mM KCl-XB buffer (stringent) and then immunoblotted. The first lane from the left was loaded with 1% of extract input. Prolonged exposures of PP2A-C and AurA immunoblots are indicated (long).
that dephosphorylation of this site may be performed by a phosphatase that promotes CDK1 activation during late interphase. 

**PP2A Associates with AurA in Xenopus Early Embryonic Extract**—Experiments using cycling egg extracts revealed that phosphatase activity restricts AurA (Thr-295) phosphorylation during two periods of an early embryonic cell cycle oscillation: one sensitive to low concentrations of OA later in interphase and another that requires more OA to permit P-AurA accumulation very early in interphase. We found that the latter activity was inhibited at OA doses insufficient to inhibit PP1, but the identity of this AurA interphase phosphatase remained unknown. Prior studies had already implicated PP2A as an AurA phosphatase in interphase (54) and on AurA (Ser(P)-51) during mitosis (37), but the recently discovered PP6 could also serve this role because it is highly sensitive to OA treatment (55, 56). However, studies to date have thus far only implicated dephosphorylation of P-AurA by PP6 in M-phase in human cells (4, 6), and the degradation of AurA during the somatic cell cycle relieves the need for this regulation during early interphase. Therefore, we focused in this study on testing whether PP2A could dephosphorylate AurA at Thr-295 under two conditions: first *in vitro* and second under *in vivo* conditions in early embryonic extract. *In vitro* phosphatase assays were done using PP2A-A/C dimer, and we compared it with the activity of recombinant PP1 (H9251)-C that was also shown to dephosphorylate P-AurA *in vitro* (9, 10, 14). Indeed, both phosphatases could dephosphorylate AurA, but in our hands, the PP2A-A/C dimer was ~30% more active toward P-AurA than PP1 (H9251)-C (Fig. 8A). Additionally, PP2A-A/C was capable of dephosphorylating AurA *in vivo* within early embryonic extract. Supplementing PP2A-A/C into cycling extract at an activity also tested in an earlier report (0.25 units of activity) (31) increased the amount of OA required to promote P-AurA accumulation, whereas adding 0.25 units of PP1 activity did not promote P-AurA dephosphorylation (Fig. 8, B and C). Last, co-immunoprecipitation of Xenopus PP2A-C and AurA together during interphase from a cycling egg extract corroborated these findings, supporting that these proteins do associate in a complex (Fig. 8D).

**PP2A-B55Δ Does Not Dephosphorylate P-AurA(Thr-295) during Early Interphase**—Co-immunoprecipitation experiments confirmed that PP2A interacts with AurA (and vice versa) during interphase in a cycling egg extract. However, this raised another question. Which endogenous B-regulatory subunit performs this function *in vivo*? Co-immunoprecipitation procedures were ineffective to identify the endogenous PP2A-A/B/C complex (data not shown), so a specific PP2A-B55Δ inhibitor, phospho-α-endosulfine (Ensa (Ser(P)-67)), was employed to test whether inhibiting B55Δ would promote P-AurA accumulation in egg extract. Phosphorylation of Ensa/Arpp19 by Greatwall kinase at Ser-67 during mitosis inhibits PP2A-B55Δ, and these proteins are well conserved among *Drosophila*, yeast, *C. elegans*, mouse, and human (5, 57). Adding phosphomimetic dEnsa (S68D) mutant into cycling extract accelerated mitotic entry, similarly to extract treated with 400 nM OA (Fig. 9A). This behavior corroborated prior findings that PP2A-B55Δ is inhibited by dEnsa (S68D), but no increase in P-AurA was detected, even in dEnsa (S68D)-treated extracts

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**FIGURE 9.** PP2A-B55Δ is not the interphase AurA phosphatase. A, time course of H1 kinase activities and protein levels in cycling extracts containing sperm chromatin (103/l) and titrated with MBP-tagged dEnsa (S68D). Extract aliquots were withdrawn at the indicated times after MBP-dEnsa (S68D) or OA addition and then immunoblotted using the indicated antibodies. The band appearing in the first lane of the cyclin B2 immunoblot (indicated by the arrowhead) in the left panel is the 50-kDa protein marker. Bars, time points where NEBD was observed. B, immunoblot of AurA (Thr(P)-295) in cycling extracts primed with MBP-dEnsa (S68D) and/or OA and sperm chromatin (103/l). Extract aliquots were withdrawn at 90 min after MBP-dEnsa (S68D) or OA addition at the indicated concentrations and then immunoblotted. Arrows, probed target; asterisks, non-specific cross-reacting band.
Cell Cycle Restriction of AurA(Thr-295) Phosphorylation

**DISCUSSION**

Numerous kinases promote the vast cellular changes of mitosis, but the roles of phosphatases are becoming more diversified and increasingly paramount. Many pathways activate the spindle-regulating kinase AurA, but its phosphorylation has been shown in our study and others to remain restricted throughout the early embryonic cell cycle. Phosphatase activity holds AurA inactive during early interphase, and here we presented evidence that PP2A may contribute to maintenance of its Thr-295 phosphorylation during that period. After phosphatase inhibition by OA, phospho-AurA(Thr-295) accumulates independently of centrosomes and CDK1 activity (Fig. 3C). Therefore, AurA activation in early embryonic extract does not need CDK1 activity per se, but with phosphatases restricting AurA phosphorylation during both interphase and mitosis, there is a clear requirement for CDK1 and other factors to activate it at mitotic entry (51).

Limiting AurA(Thr-295) phosphorylation during interphase of the early embryonic cell cycle requires phosphatase activity, but is this somehow important to cell cycle control? Indeed, primed with 100 nM OA (Fig. 9B). In summary, our use of dEnsa (S68D) excluded PP2A-B55δ as the P-AurA phosphatase. Future investigations will entail identifying the specific PP2A-B subunit that targets P-Aurora(A(Thr-295)) for dephosphorylation during interphase in early embryonic extracts as well as determining whether other OA-sensitive phosphatases serve that role.

Two P-Aurora A Phosphatases Are Required to Reproduce CDK1 Activation and Aurora A Phosphorylation in a Mathematical Model—Experiments using cycling egg extracts revealed that adding exogenous Aurora A protein with an intact Thr-295 residue would delay CDK1 activation, in correspondence with an increase in AurA(Thr-295) phosphorylation. Additionally, two different outcomes were observed, depending on the concentration of OA used; high doses of OA would accelerate CDK1 activation (as would fosstirecin), whereas very low doses of OA would delay it. This suggested that two different phosphatase activities corresponding with P-Aurora A regulation, one inhibitory and one stimulatory, might influence CDK1 activation. In order to determine whether the observed behaviors in Aurora A phosphorylation and CDK1 activation would be best recapitulated by one or two phosphatases, we tested their behaviors in a mathematical model. Using our prior ordinary differential equation model for the CDK1/APC system (53, 54), we rationalized terms for AurA function and its phosphatases (Fig. 10A and supplemental material). New variables include AurA, activated through auto- and transphosphorylation, and PP2A, which dephosphorylates P-AurA during early interphase and is inhibited by CDK1 (31). We then included in the model PPX/PP6 dephosphorylating P-AurA and inactive (phospho-)Cdc25 at a constant rate, as does PP2A-B55δ dephosphorylating active (phospho-)Cdc25 while directly (or indirectly) dephosphorylating inactive (phospho-)Wee1 (31). In contrast to the “dual phosphatase” model for AurA regulation, modeling only a single phosphatase active during both interphase and mitosis (e.g. PPX/PP6) was incapable of reproducing our experimental results (Fig. 10B, top). At a PPX/PP6 concentration of 100 nM, abnormally high levels of P-AurA were produced during CDK1 oscillations, whether without added Aurot or OA (Fig. 10B, bottom). One could argue that the concentration of PPX/PP6 in egg extract might be high enough to restrict phosphorylation of AurA, so we increased the concentration of PPX/PP6 in this “single phosphatase” model from 100 to 1000 nM. Although this increased the dephosphorylation of P-AurA slightly, there was no delay in CDK1 activation when additional Aurot or low concentrations of OA were included (Fig. 10C). This discrepancy is due to the fact that a concentration of single phosphatase that is capable of dephosphorylating P-AurA to any extent would also be sufficient to catalyze activation of all of the Cdc25. This would effectively eliminate the competitive inhibition and any delay in CDK1 activation. This issue was resolved by including PP2A along with PPX/PP6, and this “dual phosphatase” model successfully reproduced several of our experimental observations, whether after increasing AurA levels (Fig. 10D) or by decreasing PPX/PP6 activity (by including 0–400 nM OA; Fig. 10E). CDK1 activation was delayed as AurA levels were increased (Fig. 10D, left), and phospho-AurA levels increased only as AurA levels were elevated past 1 μM (Fig. 10D, right). Low OA doses (100–140 nM) also had the same effect as increases in AurA concentration (Fig. 10E). This dual phosphatase model was robust and persisted for a broad range of parameter values, keeping CDK1 activation sharp in correspondence with the mitotic delay (Fig. 10F, left). We achieved this selective inhibition of various phosphatases by OA in our model by introducing a transfer function adopted from Felix et al. (42) (Fig. 10F, right). Indeed, inhibiting both PP2A and PPX/PP6 phosphatases in the model caused a premature and shallow CDK1 activation (Fig. 10E, blue trace). Together, the results of our qualitative modeling suggest that there exists a dual phosphatase control upon AurA during an early embryonic cell cycle oscillation.

**FIGURE 10. Mathematical model coupling two phosphatases to the M-phase circuit recapitulates experimentally observed changes in CDK1 activation and P-AurA levels.** A, schematic of the CDK1 activation module (APC/proteasome portion not depicted), including known (CDK1, cyclin B, Wee1, Cdc25, PP2A-B55δ, and CDK1 inhibition of PP2A (31); shown in black) and newly proposed relationships (PP2A and PPX/PP6 (unknown phosphatase) dephosphorylating P-AurA, kinase X (unknown kinase); shown in blue). AurA autophosphorylation (auto) represents AurA phosphorylating itself; AurA transphosphorylation (trans) represents phospho-AurA phosphorylating unphosphorylated AurA. B, model output with a small contribution of PPX/PP6 alone and increasing abundance of AurA added (black, 1 μM; red, 2 μM; green, 3 μM; blue, 4 μM), plotting CDK1 activity and percentage of phosphorylated AurA (left); model output with increasing concentrations of OA added (black, 0 nM; red, 100 nM; green, 140 nM; blue, 400 nM), plotting CDK1 activity and percentage of phosphorylated AurA (right). Note the delays in CDK1 activation but lack of P-AurA dephosphorylation. C, outputs of model as stated in B but with increased PPX/PP6 (1000 nM). Note the lack of delays in CDK1 activation and only small decrease in P-AurA dephosphorylation. D, model output with both PPX/PP6 and PP2A contributing to P-AurA dephosphorylation, with an increasing abundance of added AurA (black, 1 μM; red, 2 μM; green, 3 μM; blue, 4 μM); CDK1 activity (left) and percentage of phosphorylated AurA (right) are plotted. E, model in D with increasing concentrations of OA added (black, 0 nM; red, 100 nM; green, 140 nM; blue, 400 nM), plotting CDK1 activity (left) and percentage of phosphorylated AurA (right). F, robustness analysis of mitotic delay phenotype of model shown in D (left) and transfer functions of percentage activities of PP2A-B55δ, PP2A, and PPX/PP6 incorporated in the model shown in D in response to increasing concentrations of OA (right).
adding WT or kinase-dead recombinant AurA variants in AurA-immunodepleted extracts delayed mitotic entry, but the kinase-dead AurA with Thr-295 intact (T281D) caused the longest delays in both non-depleted and mostly depleted cycling extract (Fig. 7). Although our results contrasted with the view that more AurA accelerates mitotic onset in Xenopus egg extract (43), they agreed with prior studies reporting that AurA overexpression arrests the cell cycle in human cells (27–29). Adding AurA to cycling extract during late interphase was reported to accelerate mitotic entry (43), but this neither accelerated nor delayed CDK1 activation in our experiments (Fig. 6A). This suggested that additional P-AurA(Thr-295) must be present earlier in the cycle to cause the observed M-phase delay.

Our data reveal that one or more phosphatases restrict phosphorylation of AurA(Thr-295) throughout the course of an early embryonic cell cycle. We propose that additional AurA(Thr-295) may exacerbate a normal functional competition for these phosphatase activities. This would consequently allow an increase in P-AurA, delay CDK1 activation, and possibly disrupt other events, including mitotic exit, all of which we observed in our study. This could explain why overexpressing wild-type or inactive AurA produced similar phenotypes in past studies (27, 29). This delay did not necessarily result from a nonspecific interaction with increased levels of P-AurA because additional AurA protein was not required to induce this phenotype; treating cycling extract with low concentrations of OA increased AurA(Thr-295) phosphorylation only later during interphase and delayed CDK1 activation. We identified PP2A as a likely early interphase AurA phosphatase and excluded PP1, but the latter result above suggests that it might not function alone. Partnering with PP2A could likely be PP6, an AurA(Thr-295) phosphatase that is even more sensitive to OA than PP2A (52), which dephosphorylates P-AurA during mitosis in somatic cells (4, 6). PP6-depleted cells cannot form a bipolar spindle with normal kinetics and fail to align chromosomes efficiently, demonstrating its mitotic function (4, 6). However, these studies did not address the role of AurA regulation by PP6 during interphase, and it is possible that it could serve to coordinate AurA kinase and CDK1 activation prior to M-phase onset. This could be especially important to the early embryonic cell cycle oscillator, where AurA is not degraded at mitotic exit, and restriction of its phosphorylation must occur immediately in interphase. Although we identified PP2A as a likely early interphase P-AurA phosphatase, future work is aimed at determining if PP6 also contributes to this regulation during the embryonic cell cycle. Phosphatase activities target the AurA(Thr-295) residue and appear to have an influence over the timing of mitotic entry during late interphase. It is possible that the PP2A isoform that dephosphorylates AurA during early interphase is inhibited or inaccessible to AurA during late interphase and requires the activity of a phosphatase such as PP6. Indeed, our mathematical modeling supported this idea, revealing that function of a single P-AurA phosphatase (such as PP2A or PP6) would not cause the observed experimental changes in AurA phosphorylation and that at least two phosphatases (possibly both PP2A and PP6) are necessary. Apart from PP2A dephosphorylating AurA during interphase, the second phosphatase (PPX/PP6) may play the same role during late interphase and mitosis while also removing an inhibitory phosphate from Cdc25 before its activation by CDK1. Alternatively, it could also remove an activating phosphate from Wee1. We hypothesize that the continual auto- and transphosphorylation of AurA provides substrate for PPX/PP6 during late interphase, resulting in “competitive inhibition” between two substrates, P-AurA and P-Cdc25 (Fig. 10A), and that this would prevent the accumulation of significant levels of P-AurA during a cell cycle oscillation.

In summary, we propose that a differential restriction of AurA phosphorylation occurs during an early embryonic cell cycle oscillation by at least one early interphase AurA phosphatase (PP2A) and one that functions prior to mitotic onset (possibly PP6). Based upon published examples of pathogenic conditions altering AurA abundance, these are plausible connections. For example, AurA gene amplification and/or protein overexpression correlates with many cancers, and increases in protein/activity are thought to underlie oncogenesis (7). Prior work revealed that PP2A dephosphorylation of AurA(Ser-51) (Xenopus Ser-53) promotes AurA degradation in somatic human cells (37), and our study reveals another PP2A function seemingly important for the early embryonic cell cycle: restricting interphase AurA activity by limiting its Thr-295 phosphorylation. These studies together further underscore that mutations and/or defects in the phosphatases that regulate AurA phosphorylation and activity might not simply impede proper mitotic function but could also affect cell cycle timing.

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