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NDEL1 Phosphorylation by Aurora-A Kinase Is Essential for Centrosomal Maturation, Separation, and TACC3 Recruitment▽†

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NDEL1 is a binding partner of LIS1 that participates in the regulation of cytoplasmic dynein function and microtubule organization during mitotic cell division and neuronal migration. NDEL1 preferentially localizes to the centrosome and is a likely target for cell cycle-activated kinases, including CDK1. In particular, NDEL1 phosphorylation by CDK1 facilitates katanin p60 recruitment to the centrosome and triggers microtubule remodeling. Here, we show that Aurora-A phosphorylates NDEL1 at Ser251 at the beginning of mitotic entry. Interestingly, NDEL1 phosphorylated by Aurora-A was rapidly downregulated thereafter by ubiquitination-mediated protein degradation. In addition, NDEL1 is required for centrosome targeting of TACC3 through the interaction with TACC3. The expression of Aurora-A phosphorylation-mimetic mutants of NDEL1 efficiently rescued the defects of centrosomal maturation and separation which are characteristic of Aurora-A-depleted cells. Our findings suggest that Aurora-A-mediated phosphorylation of NDEL1 is essential for centrosomal separation and centrosomal maturation and for mitotic entry.

Centrosomes are a morphologically diverse group of organelles that share a common ability to nucleate and organize microtubules (MTs) and are thus referred to as microtubule-organizing centers (7). Centrosomes order the interphase cytoplasm and play a major role in establishing the structure of the mitotic spindle. To accomplish these tasks, the replication and microtubule-nucleating capacity of the centrosome must be precisely regulated during the cell cycle (29, 34). The duplication of the centrosome initiates around the time of S phase and is completed before mitosis so that two centrosomes are available to form the poles of the bipolar spindle. Duplication involves the splitting of a centriole pair, and this is followed by the synthesis of a new centriole adjacent to each preexisting centriole (21). The replicated centrosomes then migrate apart in preparation for spindle assembly. As cells progress toward mitosis, the size and microtubule-nucleating capacity of the centrosome increase, a process termed maturation (29). Maturation involves the recruitment of pericentriole material components, such as γ-tubulin (35) and pericentrin (6).

This dynamic morphological change of centrosomes at the progression to mitosis is a tightly regulated cellular process that requires the activation of the CDK1 kinase, which determines the onset of mitosis in all eukaryotic cells. Recent studies have brought to light additional mitotic kinases, which include members of the polo family, the NIMA (never in mitosis A) family, and the Aurora family (27, 30).

Aurora kinases compose a family of serine/threonine kinases whose multiple roles within each subfamily are conserved throughout evolution. The founding member of this protein family, Aurora-A, originally derived its name from a *Drosophila* mutant displaying morphological defects at the mitotic spindle pole, reminiscent of an aurora, a night sky phenomenon in polar regions (13). Homologs have since been identified in various species, with a single gene in budding and fission yeasts and up to three genes, Aurora-A, -B, and -C, in mammals (2, 11). Among Aurora kinases, Aurora-A begins to accumulate on centrosomes during S phase, and by mitosis, it is heavily concentrated on centrosomes at the spindle poles in addition to being detectable along spindle microtubules. Aurora-A plays an essential role in centrosome maturation in various organisms. In *Caenorhabditis elegans*, RNA interference-mediated silencing of Aurora-A results in a decreased density of centrosome-associated MTs, a decreased accumulation of

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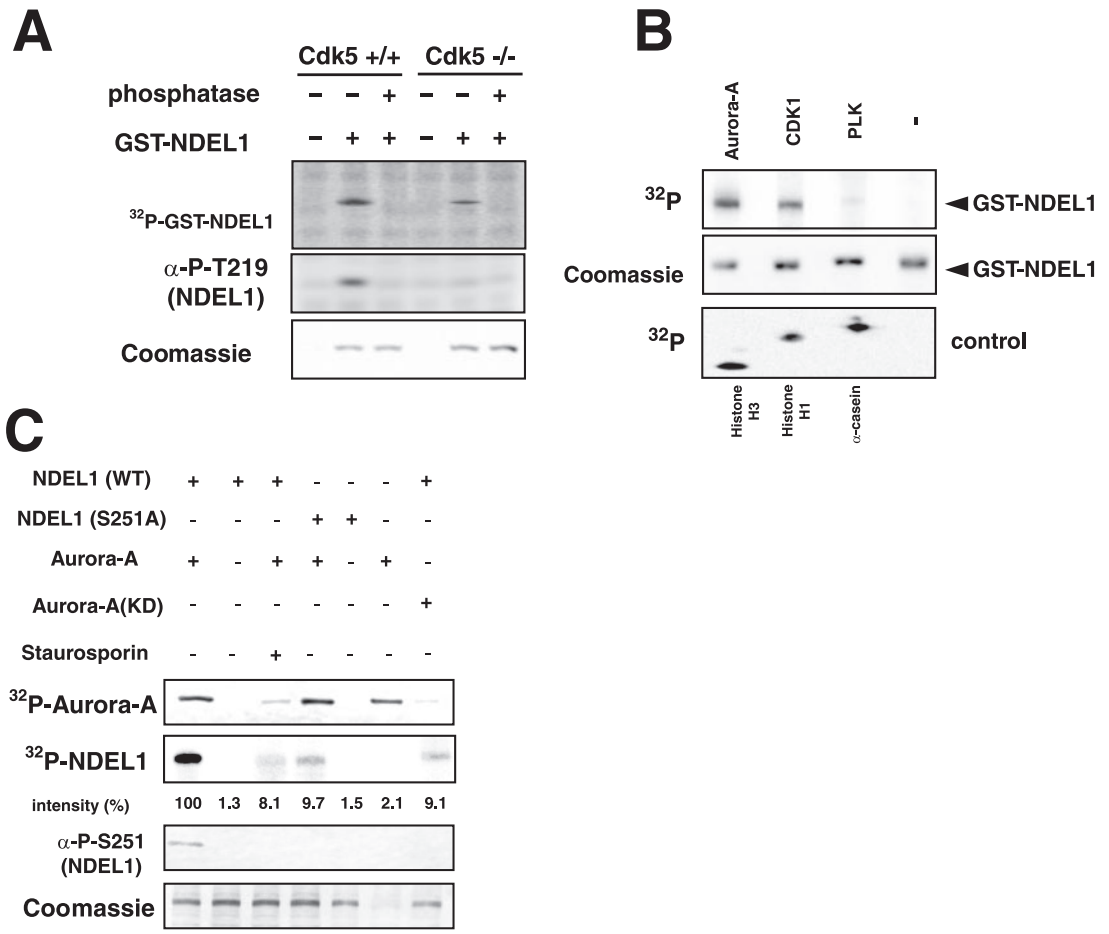


FIG. 1. Aurora-A kinase phosphorylates Ser251 of NDEL1. (A) We performed in vitro phosphorylation using brain lysates from E15.5 wild-type mice or $Cdk^{-/-}$ mice (28). Phosphorylation was detected by the incorporation of ^{32}P (upper panel) or by an anti-phospho-T219 (α-P-T219) monoclonal antibody (middle panel) (38). Phosphorylation at T219 was undetectable in lysates from $Cdk^{-/-}$ mice, but the lysate was competent to phosphorylate NDEL1 at other sites. Note that phosphatase removed this phosphorylation. (B) In vitro kinase assays using several mitotic kinases that are localized at the centrosome. CDK1 and Aurora-A efficiently phosphorylated NDEL1. Histone H3, histone H1, and casein were used as a positive control for each kinase. A control panel for protein loading is shown at the middle panel. (C) Examination of the effect of mutation at S251 or an Aurora-A inhibitor, staurosporin, on phosphorylation. Aurora-A(T288A) [Aurora-A(KD)] was used as a negative control of Aurora-A. The relative intensity of phosphorylation is described at the bottom. Phosphorylation was detected by the incorporation of ^{32}P or an anti-phospho-S251 monoclonal antibody (see below). Either mutation or staurosporin significantly reduced phosphorylation. Note, an inactive form of Aurora-A, Aurora-A(KD), resulted in only background phosphorylation of NDEL1. −, absence of; +, presence of.

γ-tubulin, and a failure to maintain centrosome separation in mitosis (15). Similarly, in *Drosophila*, Aurora-A mutation or RNA interference leads to a reduction in the length and density of astral MTs (1, 12). Centrosomes also appear to be disorganized, with less focused MT arrays, abnormal centriole numbers, occasional failures in centrosome separation, and a reduced recruitment of pericentriole material proteins, including γ-tubulin and Minispindles, an XMAP215 homolog in *Drosophila* (1, 10, 22, 36, 37). Although these molecules are known to be downstream of Aurora-A, how they promote centrosome maturation and separation is largely unknown.

We found that NDEL1, a LIS1-interacting protein, is a substrate of Aurora-A. *LIS1* was identified as a mutated gene in the isolated lissencephaly sequence (31); lissencephaly is a cerebral cortical malformation characterized by a smooth cerebral surface and a disorganized cortex (4, 5) due to incomplete neuronal migration. LIS1 protein is highly conserved from humans to *Aspergillus* (24, 25). The LIS1 homologue in *Aspergillus*, *nudF* (41), was originally identified as a gene mutated in a series of hyphal mutants exhibiting defects in nuclear migration. We and others previously reported that there are two mammalian homologues of *Aspergillus* NudE, NDE1 (8) and NDEL1 (26, 32). NDEL1 participates with LIS1 in the regulation of cytoplasmic dynein heavy chain function via phosphorylation by CDK5/p35 (8, 26, 32), a complex known to be essential for neuronal migration (14). *Lis1*-disrupted mice and *Ndel1*-disrupted mice displayed similar neuronal migration defects (17, 32). In addition, null mutations of each gene result in early embryonic lethality (17, 33), supporting the hypothesis that LIS1 and NDEL1 participate in a common molecular pathway. Interestingly, NDEL1 is a known substrate of several kinases, including CDK5 (at Ser198, Thr219, and Ser231 [see below]), which are essential for the regulation of proper MT organization (38). In particular, we demonstrated that CDK5/

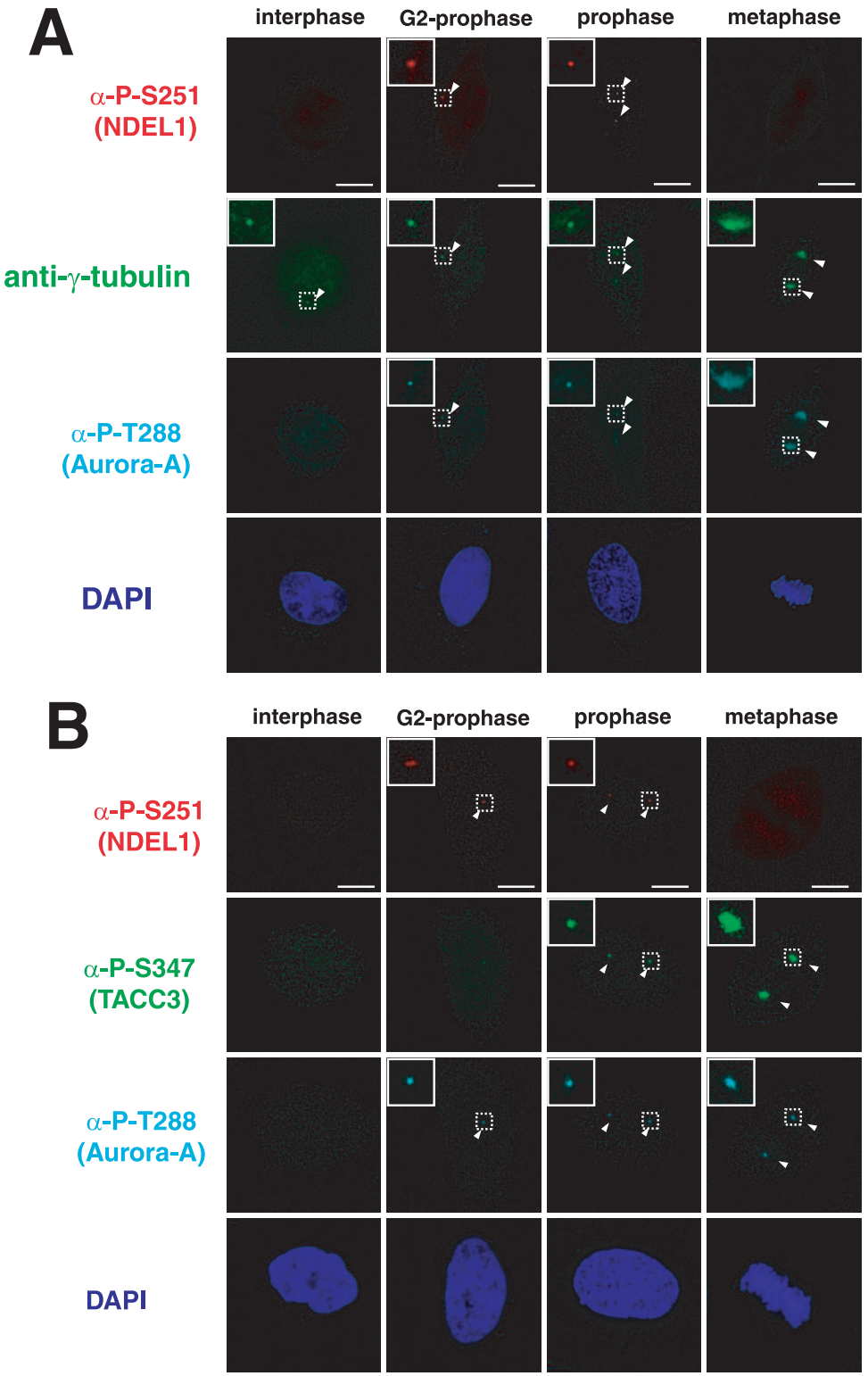
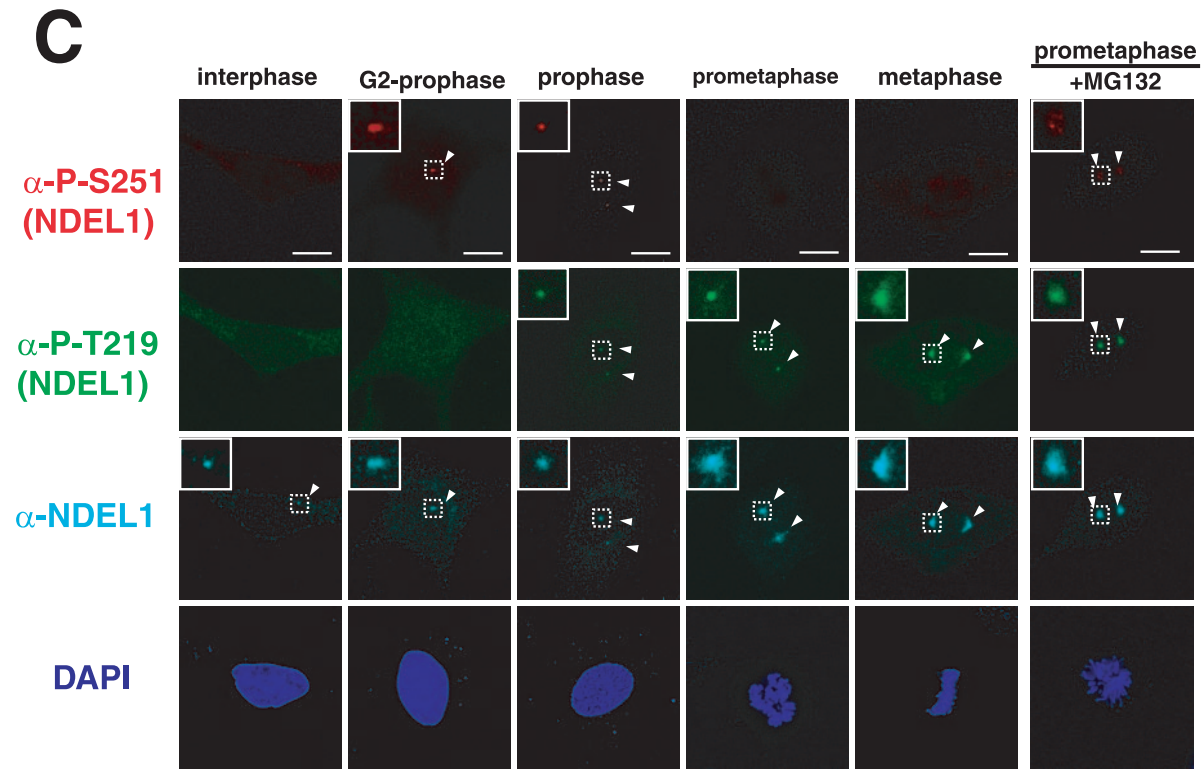


FIG. 2. Characterization of Ser251 phosphorylation of NDEL1 by Aurora-A using an anti-phosphorylated Ser251-specific monoclonal antibody. HeLa cells synchronized in G_1/S with a double-thymidine block were used in these experiments. (A) Characterization of S251 phosphorylation of NDEL1 using an anti-phospho-S251-specific monoclonal antibody. The activation of Aurora-A was determined by the phosphorylation of T288. The phosphorylation of S251 appeared at G_2 prophase, coincident with Aurora-A activation. Although Aurora-A was active during mitosis, the phosphorylation of S251 was rapidly downregulated after prophase. (B) S347 phosphorylation of TACC3 appeared at the onset of prophase as previously reported (19), which inversely correlated with the phosphorylation of NDEL1 (see below). (C) Examination of S251 phosphorylation and T219 phosphorylation. Compared to S251 phosphorylation, T219 phosphorylation appeared at prophase and continued throughout mitosis. Rapid downregulation of S251 phosphorylation was prevented by the administration of MG132 (right panels). (D) HeLa cells



synchronized in G_1/S with double-thymidine block were lysed after the release of the block at the indicated time, followed by Western blotting using each antibody. S251 phosphorylation displayed a peak at 8.5 h after release, which preceded by 0.5 h the appearance of T219 phosphorylation. The administration of MG132 prevented the degradation of phosphorylated NDEL1 at S251. In contrast, OA was not effective in preventing its reduction. An arrowhead indicates the point of administration of MG132 or OA. The relative intensity of Western blot signals is summarized at the bottom. Standard errors were calculated from the results of three independent experiments. Error bars indicate standard deviations. (E) In vitro kinase assay using wild-type GST-NDEL1, GST-NDEL1(tripleS/T→E), and GST-NDEL1(tripleS/T→A) (see Materials and Methods) by Aurora-A. Phosphorylation activity by Aurora-A to NDEL1s was determined by an incorporation of ^{32}P or by Western blotting using an anti-phospho-S251-specific monoclonal antibody. Significant reduction of the phosphorylation was observed in GST-NDEL1(tripleS/T→E). Note that the GST-NDEL1(tripleS/T→E) band displayed an upward shift of the band compared to that for other proteins. (F) In vitro kinase assay using wild-type GST-NDEL1, GST-NDEL1(S251E), and GST-NDEL1(S251A) (see Materials and Methods) by CDK1. Phosphorylation activity by CDK1 NDEL1s was determined by an incorporation of ^{32}P or by Western blotting using the anti-phospho-T219-specific monoclonal antibody. There was no obvious difference in the kinase activities of the proteins. Note that GST-NDEL1(S251E) displayed the same mobility as that of other proteins. α, anti.

CDK1-mediated phosphorylation of NDEL1 recruits katanin p60 at the centrosome and facilitates MT remodeling (38).

An examination of the phosphorylation status of NDEL1 in *Cdk5*-null mutants suggested that NDEL1 may be a substrate of other kinases. To discover other NDEL1 kinases, we systematically surveyed the phosphorylation of NDEL1 by using liquid chromatography-atmospheric pressure ionization tandem mass spectrometry (LC-MS/MS) and found a novel phosphorylation site (S251) of NDEL1. Using a candidate approach, we demonstrated that Aurora-A kinase efficiently phosphorylated S251 of NDEL1. S251 phosphorylation by Aurora-A commenced at the beginning of prophase, which coincides with centrosome maturation. Interestingly, NDEL1 is initially phosphorylated by Aurora-A, followed by rapid down-regulation of phosphorylated NDEL1, whereas the phosphorylation of NDEL1 by CDK1 starts at the prophase-metaphase transition and continues during mitotic events. We also found that NDEL1 binds TACC3, which is another Aurora-A target, with extremely strong affinity (9, 19). *Ndel1* disruption severely impaired the recruitment of TACC3 to the centrosome despite the presence of active Aurora-A, suggesting that NDEL1 is required for centrosome targeting of TACC3. While Aurora-A-depleted cells displayed an impairment of centrosome separation and maturation defects, these phenotypes were efficiently rescued by the exogenous expression of a phosphorylation-mimetic mutant of NDEL1, resulting in a recovery of mitotic entry. Our findings suggest that NDEL1 is a key molecule that connects Aurora-A to other downstream molecules during mitosis.

MATERIALS AND METHODS

In vitro phosphorylation of NDEL1 by brain extracts. Cortices were dissected from wild-type mice or $Cdk5^{-/-}$ mice (28) at embryonic day 15.5 (E15.5), and soluble protein was extracted with 20 mM K-HEPES (pH 7.4), 50 mM KCl, 1 mM dithiothreitol (DTT), 0.2% NP-40, and protease inhibitor cocktail (Roche). The protein concentration was adjusted to 100 μg/ml and used for in vitro kinase reactions. In vitro kinase reactions were carried out using 1 μg of recombinant NDEL1 and 0.5 μg of brain extract protein expressed in bacteria under the condition of 20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol with 10 μCi of [γ-^{32}P]ATP. Phosphorylation was detected by ra-

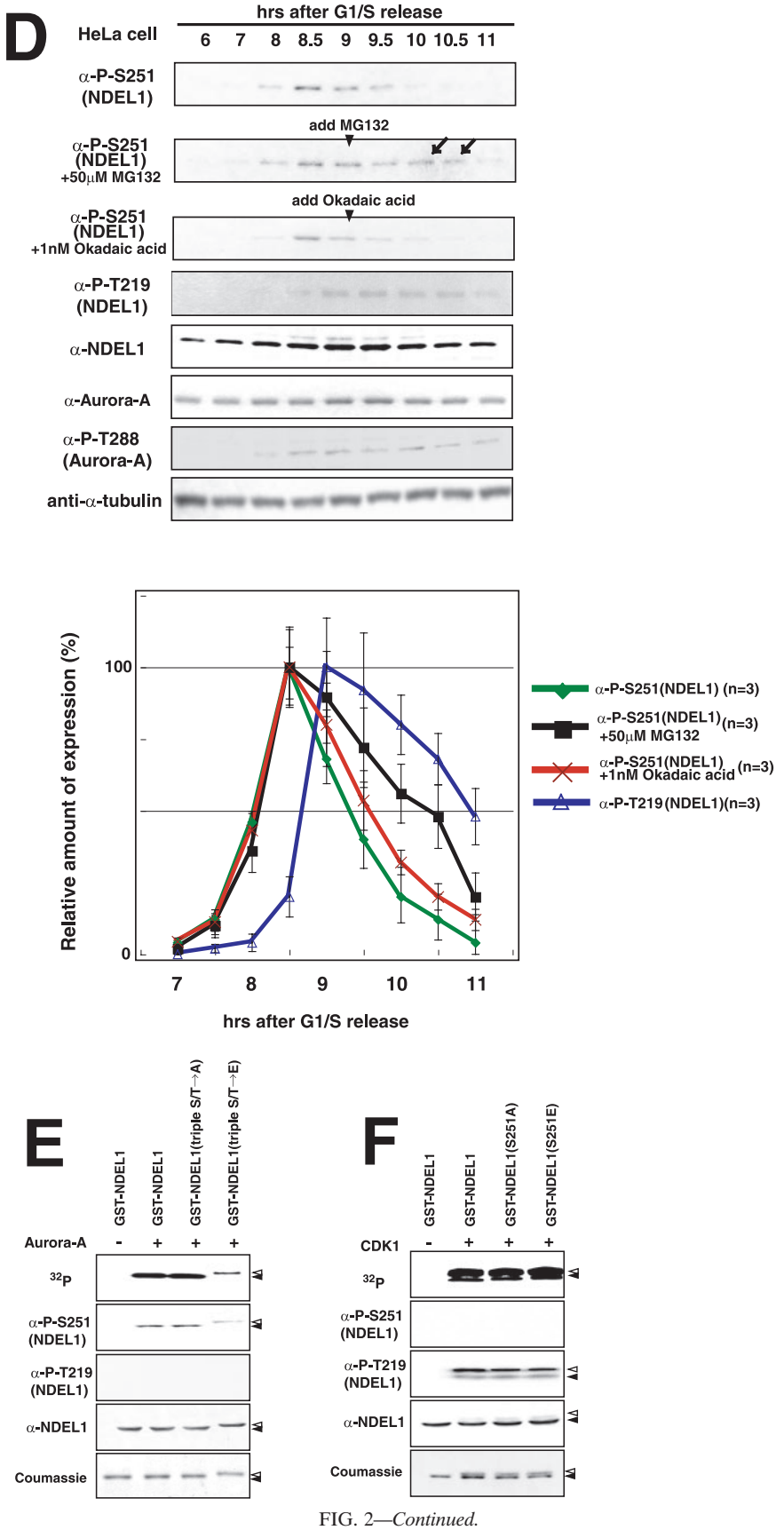


FIG. 2—*Continued.*

dioactivity or by Western blot analysis using an anti-phospho-T219 monoclonal antibody (38).

Generation of *Ndel1* stable transformants and replacement experiments. We established mouse embryonic fibroblasts (MEFs) from *Ndel1*$^{cko/cko}$ mice (33). We cotransfected the *Cre* expression vector and the glutathione *S*-transferase (GST)-NDEL1 expression vector, which carried a Neo^r gene (Clontech), and followed this by selection with G418. *Ndel1*-null MEFs are nonviable, whereas GST-NDEL1-transformed MEFs are viable and grow normally. We also examined the expression amounts and subcellular localization by Western blot analysis using the anti-NDEL1 antibody (32) and an anti-GST antibody (Upstate). GST-NDEL1 was purified by GST-Sepharose 4B (Amersham) based on the manufacturer's recommended conditions.

For replacement experiments, we transfected expression vectors that carry green fluorescent protein (GFP)-tagged wild-type NDEL1 and mutated NDEL1s (see below) to MEF cells in which endogenous *Ndel1* was removed by CRE-mediated recombination before synchronization. MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma) containing 10% fetal bovine serum. For synchronization, G_1 cells were obtained by culturing cells in DMEM with 0.1% newborn calf serum for 2 days. After release, MEFs were fixed or harvested at a given time.

Identification of phosphorylation site of NDEL1 by Aurora-A. To determine native phosphorylation sites of NDEL1, GST-NDEL1 was extracted from 8×10^7 MEF cells in which endogenous *Ndel1* was replaced by *GST-Ndel1* (see above) using lysis buffer (20 mM K-HEPES, pH 7.4, 50 mM KCl, 1 mM DTT, 0.2% NP-40, protease inhibitor cocktail [Roche], 1 mM EDTA, 1 mM sodium orthovanadate, and 1 mM NaF) and purified using GST-Sepharose (Amersham). Purified GST-NDEL1 (0.5 μg) was digested with trypsin (Roche), and this was followed by the determination of phosphorylation sites using liquid chromatography/atmospheric pressure ionization tandem mass spectrometry with a hybrid triple-quadruple linear ion trap mass spectrometer (QTrap) (ABI) based on the manufacturer's manual. We performed a series of experiments with three independent cell lysates and obtained the same results in each case.

Generation of recombinant protein and in vitro kinase reactions. We generated GST-tagged, full-length recombinant Plk1 by using a Bac-to-Bac baculo system (Invitrogen) with SF-9 or High Five insect cells (Pharmingen). To obtain active Plk1, insect cells were cultured in the presence of 100 nM okadaic acid (OA) (Sigma) in dimethyl sulfoxide 3 h prior to harvesting. Forty-eight hours postinfection, Plk1 proteins were purified as described previously (32) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or used for kinase assays. Active Aurora-A was expressed in insect cells and purified as reported previously (23). CDK1 was purchased from New England Biolabs. In vitro kinase reactions were performed in the following conditions with 10 μCi of [γ-^{32}P]ATP: for Cdk1 (New England Biolabs), 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM EGTA, 2 mM dithiothreitol, and 0.01% Brij 35; for Plk1, 20 mM HEPES (pH 7.7), 15 mM KCl, 10 mM $MgCl_2$, 1 mM EGTA, and 5 mM NaF at 37°C for 30 min; and for Aurora-A (23), 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol. A total of 0.4 μg of each purified kinase and 1 μg of histone H3 were used in each 50-μl reaction mixture. The reaction was stopped by the addition of Laemmli sample buffer, and phosphorylated substrates were detected by SDS-PAGE and autoradiography. Staurosporin (100 nM; CycLex) was used as an inhibitor of Aurora-A.

As a substrate, we generated recombinant NDEL1 using the pGEX (Amersham) vector to express in the bacteria. To generate structurally more natural recombinant proteins for the sucrose gradient sedimentation assay and the Biacore assay, we generated GST-tagged, full-length recombinant NDEL1, TACC3, and mutant proteins by using the Bac-to-Bac baculo system (Invitrogen) with SF-9 or High Five insect cells (Pharmingen). Protein purification was performed using GST-Sepharose (Amersham) based on the manufacturer's recommendations.

Generation of NDEL1 expression vectors. A PCR fragment of full-length *Ndel1* was cloned into Bluescript II (Stratagene) and sequenced. The XhoI-XbaI fragment excised from the original vector was cloned into the Bac-to-Bac baculo system or pEGFP (Clontech) for expression in insect cells and mammalian cells, respectively. For the expression in mammalian cells, we replaced the AseI-AgeI fragment carrying the cytomegalovirus promoter of pEGFP with the HindIII-HindIII fragment carrying the elongation factor promoter (EF-1α) from pEF6/Myc-His (Invitrogen) for gradual and mild expression. Mutated NDEL1 constructs in which phosphorylation sites were changed to alanine or glutamic acid were generated by QuikChange (Stratagene). These constructs contain the following mutations: NDEL1(S251A), S251A; NDEL1(S251E), S251E; NDEL1(tripleS/T→A), Ser198A, Thr219A, and Ser231A; NDEL1(tripleS/T→E), Ser198E, Thr219E, and Ser231E; NDEL1(tripleS/T→A;S251E), Ser198A, Thr219A, Ser231A and S251E; and NDEL1(tripleS/T→E;S251E), Ser198E, Thr219E, Ser231E and S251E. Transformation was performed with a Gene Pulser (Bio-Rad) based on the manufacturer's recommended conditions.

Generation of an anti-Ser251 monoclonal antibody. Six-week-old BALB/c mice were immunized with 50 μg of keyhole limpet hemocyanin-conjugated oligopeptide [CTPSARIS(PO_3H_2)ALN] in Freund's complete adjuvant (Difco), with a second injection in Freund's incomplete adjuvant (Difco) 14 days later. The splenocytes of the immunized mouse were fused to SP2/0 mouse myeloma cells by using a standard polyethylene glycol protocol. The fused cell population was resuspended in hypoxanthine aminotropterin thymidine selection medium (Life) and plated into 96-well, flat-bottomed culture plates. Positive clones that specifically recognized phosphorylated NDEL1 were subcloned to obtain clonal hybridomas. Subtyping of our anti-phosphorylated NDEL1 monoclonal antibody revealed immunoglobulin M, which makes it possible to perform double staining with another anti-phosphorylated NDEL1 monoclonal antibody (38).

Ubiquitination assay. For immunoprecipitation, wild-type GFP-NDEL1 and mutant NDEL1s were transfected to synchronized HeLa cells. Cells were lysed at given times by lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% NP-40, and protease inhibitor cocktail; Roche) and incubated with an anti-GFP rabbit polyclonal antibody (Clontech) in 100 μl, followed by immunoprecipitation. Immunoprecipitates were subjected to SDS-PAGE analysis, followed by immunoblotting using an anti-Ub mouse monoclonal antibody (Santa Cruz; catalog no. sc-8017).

Synchronization of HeLa Cells, siRNA, and immunohistochemistry. HeLa cells were cultured in modified Eagle's medium (MEM) (Sigma Chemical Co.) supplemented with 10% fetal calf serum. For mitosis synchronization, HeLa cells were exposed to 2 mM thymidine for 16 h and then resuspended in fresh medium supplemented with 24 μM 2′-deoxycytidine and allowed to grow for 9 h. Thymidine (2 mM) was added again for 16 h, causing cells to accumulate near the G_1/S boundary (23). After release from the double-thymidine block, cells were harvested at given times. The sequences of the short interfering RNAs (siRNAs) were as follows: for human Aurora-A, 5′-AUUCUUCCCAGCGCGUUCC-3′ (corresponding to nucleotides 155 to 173 relative to the start codon) or 5′-AU GCCCUGUCUUACUGUCA-3′ (nucleotides 725 to 743) and, for human TACC3, 5′-GUU ACC GGA AGA UCG UCU GTT-3′ (nucleotides 85 to 103) or 5′-CAG ACG AUC UUC CGG UAA CTT-3′ (nucleotides 103 to 85) (19). For the inhibition of phosphatase and proteasome, we treated synchronized HeLa cells with okadaic acid and MG132, respectively. To minimize the secondary effect of these inhibitors, we optimized the dosage and duration of the treatment. Briefly, 1 nM of okadaic acid and 50 μM of MG132 were added at a given time for 30 min before harvesting for Western blotting or immunocytochemistry. Western blot analysis was performed for three independent cell lysates, and the standard error was calculated.

For rescue experiments, siRNA and each NDEL1 construct were introduced into HeLa cells simultaneously using Oligofectamine (Invitrogen). HeLa cells were grown in 35-mm petri dishes, fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 20 min at room temperature, and permeabilized with 0.5% Triton X-100 in phosphate-buffered saline. After incubation for 60 min with 5% goat serum in phosphate-buffered saline, the cells were incubated at 4°C overnight with primary antibodies and then for 45 min at room temperature with fluorescein isothiocyanate-, Cy5-, or rhodamine-conjugated secondary antibodies (Bio-source, Molecular Probes, or Pharmacia).

Measurement of mitotic index. Synchronization and transfection of HeLa is described above. Cells were observed 8.5 h after release by phase-contrast microscopy. The time course of the mitotic index of cells was measured at the indicated time, and the number of mitotic cells out of 1,000 cells examined in total was determined after fixation and staining with DAPI (4′,6′-diamidino-2-phenylindole).

Surface plasmon resonance. Real-time biospecific interaction analysis was performed using a Biacore 2000 (Pharmacia Biosensor). Each protein was immobilized to CM5 biosensor microchips using *N*-hydroxy-succinimide and *N*-ethyl-*N*′-dimethylaminopropyl carbodiimide. In standard surface plasmon resonance experiments, kinetic binding experiments were performed under previously reported conditions (38). The same running buffer was used (50 mM KCl, 20 mM HEPES, pH 7.4, 1 mM DTT, and 0.1% NP-40), and the instrument was equilibrated at 24°C. Kinetic data were collected by injecting various concentrations of TACC3, CH-TOG/XMAP215, NDEL1, and mutated NDEL1s. Kinetic rate constants were determined by fitting the corrected response data to a simple bimolecular interaction model, A + B = AB. Kinetic constants K_A and K_D are the association constant and dissociation rate constant, respectively.

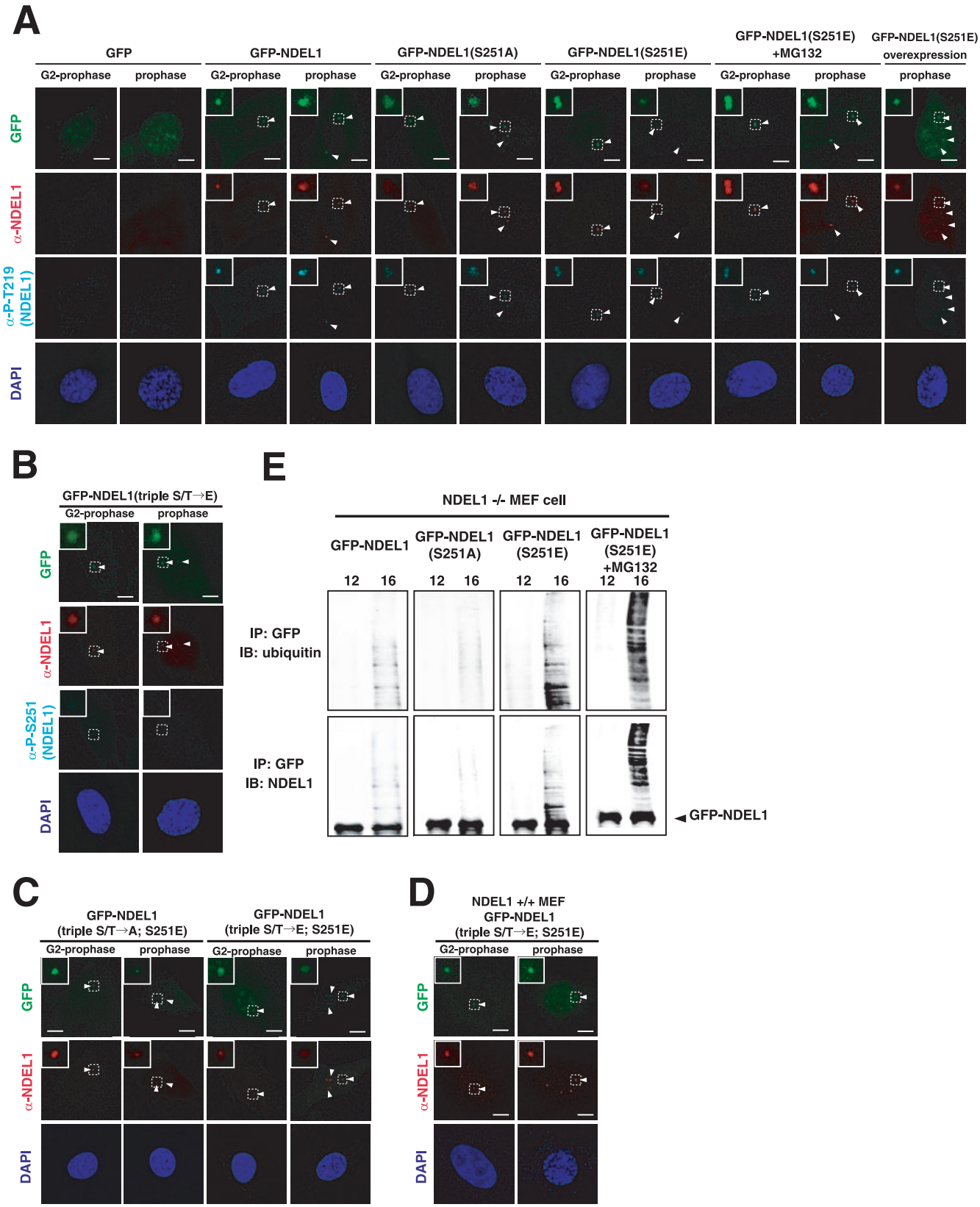


FIG. 3. Ser251 phosphorylation of NDEL1 by Aurora-A facilitates centrosomal targeting of NDEL1 and ubiquitin-mediated protein degradation. (A) Replacement experiment of endogenous NDEL1 by wild-type GFP-NDEL1, GFP-NDEL1(S251A), or GFP-NDEL1(S251E) with/without MG132. Wild-type GFP-NDEL1 displayed normal localization. In contrast, GFP-NDEL1(S251A) displayed diffuse distribution throughout the cell cycle, whereas GFP-NDEL1(S251E) displayed more restricted localization at the centrosome in G_2 prophase, followed by its

RESULTS

Identification of a novel phosphorylation site of NDEL1 by Aurora-A kinase. We and other groups found that NDEL1 is a substrate of Cdk5/p35 (26, 32), and Cdk5/p35-mediated phosphorylation was lost in *Cdk5*-null mice (26). We extracted brain lysates from E15.5 $Cdk5^{-/-}$ mice or wild-type mice and performed in vitro kinase assays to examine whether the $Cdk5^{-/-}$ lysates lost competence to phosphorylate NDEL1. As expected, lysates derived from $Cdk5^{-/-}$ mice lost the ability to phosphorylate consensus sites (at Ser198, Thr219, and Ser231) of CDK5/CDK1 on GST-NDEL1, but GST-NDEL1 was still efficiently phosphorylated, suggesting that NDEL1 was a substrate of another kinase (Fig. 1A). To hunt for novel kinases of NDEL1, we established a stable MEF line in which endogenous NDEL1 was replaced by GST-tagged exogenous NDEL1 (33). In this MEF line, GST-NDEL1 was expressed in normal amounts and distributed normally compared to endogenous NDEL1 (see Fig. S1A and S1B in the supplemental material), suggesting that the protein could be phosphorylated by the same kinase as endogenous NDEL1. Next, we purified GST-NDEL1 (see Fig. S1C in the supplemental material) and performed a systematic analysis of phosphorylation sites using LC-MS/MS. We found several phosphorylation sites in MEFs, including T219 and S231, which are thought to be phosphorylation sites for CDK1/CDK5 (26, 32) (see Fig. S1D in the supplemental material). We also found an additional phosphorylation site, S251 (see Fig. S1D in the supplemental material). Interestingly, S251 phosphorylation was observed only when GST-NDEL1 was extracted from dividing MEFs and was undetectable when GST-NDEL1 was isolated from stationary MEFs (data not shown), suggesting that this novel phosphorylation of S251 may be attributed to a mitotic kinase.

NDEL1 is a centrosomal protein. Therefore, we considered several mitotic kinases that are preferentially localized at the centrosome as candidates to phosphorylate recombinant NDEL1. As expected, CDK1 efficiently phosphorylated NDEL1 (26, 32) (Fig. 1B). We also confirmed by LC-MS/MS that CDK1 phosphorylated T219 and S231, whereas the phosphorylation of S198 was less efficient (data not shown). Plk1 is another kinase that functions during mitosis (27), but even when using okadaic acid for activation of Plk1 (18, 20), recombinant NDEL1 was not phosphorylated (Fig. 1B). Finally, we examined Aurora-A kinase and found that Aurora-A efficiently phosphorylated recombinant NDEL1 expressed from insect cells (23) (Fig. 1B). S251 and the surrounding amino acid sequence were weakly compatible with the consensus sequence of budding yeast protein kinase Ipl1/Aurora {KR}X{TS}{ILV} (3). We subjected NDEL1 phosphorylated by Aurora-A to LC-MS/MS and confirmed that Aurora-A phosphorylated S251 (data not shown). Next, we examined the effect of the mutation of S251 or an inhibitor of Aurora-A on phosphorylation. The replacement of serine by alanine at S251 or the presence of Aurora-A inhibitor staurosporin (39) significantly reduced the phosphorylation by Aurora-A (Fig. 1C). In addition, an introduction of the mutation at the catalytic site of Aurora-A significantly reduced the phosphorylation of NDEL1 (Fig. 1C). Thus, we concluded that Aurora-A specifically phosphorylates S251 of NDEL1. Direct interaction with Aurora-A appears to be essential for phosphorylation (23). We examined whether NDEL1 directly binds Aurora-A and whether the phosphorylation of NDEL1 by Aurora-A influences the interaction between the two proteins using wild-type *Ndel1* and mutated *Ndel1*s. Immunoprecipitation revealed that GFP-NDEL1(S251E) was more efficiently precipitated with Aurora-A than was GFP-NDEL1(S251A) (see Fig. S1E in the supplemental material), suggesting that phosphorylated NDEL1 binds more tightly with Aurora-A and phosphorylated NDEL1 may preferentially distribute at the centrosome (see below).

Aurora-A phosphorylates S251 of NDEL1 at the beginning of prophase, followed by rapid downregulation. To examine the phosphorylation of S251 by Aurora-A, we generated an anti-phosphorylated S251-specific monoclonal antibody (see Fig. S2 in the supplemental material) and monitored Aurora-A-mediated S251 phosphorylation through mitosis using HeLa cells. Given that the phosphorylation of Aurora-A on T288, which is located in the activation T loop of the kinase, results in a marked increase in enzymatic activity (40), we examined the phosphorylation state of T288 by immunohistochemistry with the anti-phospho-T288 antibody of Aurora-A. Consistent with previous observations (23, 40), the phosphorylation of Aurora-A on T288 occurred during the late G_2-prophase transition, coincident with enzymatic activation (Fig. 2A). As expected, S251 phosphorylation of NDEL1 appeared coincidently with Aurora-A activation. Interestingly, the activation of Aurora-A continued through mitosis and peaked at metaphase-anaphase, while S251 phosphorylation suddenly disappeared after prophase (Fig. 2A). TACC3 is another target of Aurora-A, which localizes specifically to mitotic centrosomes at the onset of prophase (9, 19) (Fig. 2B). Interestingly, the

disappearance after progression to prophase. This rapid disappearance was prevented by the administration of MG132. Interestingly, the expression of an excess of the GFP-NDEL1(S251E) mutant resulted in multipolar centrosomes rather than their disappearance and the majority of them arrested at prophase. (B) Replacement experiments of endogenous NDEL1 by GFP-NDEL1(tripleS/T→E). GFP-NDEL1(tripleS/T→E) displayed a diffuse distribution pattern like that of GFP-NDEL1(S251A). In addition, a signal was not detected by an anti-phospho-S251 antibody, supporting the interpretation that the phosphorylation by CDK1 inhibited further phosphorylation by Aurora-A. (C) Replacement experiment of endogenous NDEL1 by GFP-NDEL1(tripleS/T→A;S251E) or GFP-NDEL1(tripleS/T→E;S251E). Both mutant NDEL1s displayed centrosomal patterns, but the expression of GFP-NDEL1(tripleS/T→E;S251E) was more unstable and associated with multipolar centrosomes. (D) Expression of GFP-NDEL1(tripleS/T→E;S251E) in wild-type cells. Multipolar centrosomes were also observed in wild-type cells by expression of GFP-NDEL1(tripleS/T→E;S251E), suggesting that this phenotype may be dominant. (E) Western blot using an anti-Ub antibody and an anti-NDEL1 antibody as indicated. Synchronized MEF cells were transfected by expression vectors carrying wild-type GFP-NDEL1, GFP-NDEL1(S251A), or GFP-NDEL1(S251E), and this was followed by immunoprecipitation (IP) at a different time (indicated above panel). Wild-type GFP-NDEL1 was preferentially ubiquitinated at prophase (16 h). Note the decreased ubiquitination of GFP-NDEL1(S251A), whereas enhanced ubiquitination was observed with GFP-NDEL1(S251E). IB, immunoblot; α, anti.

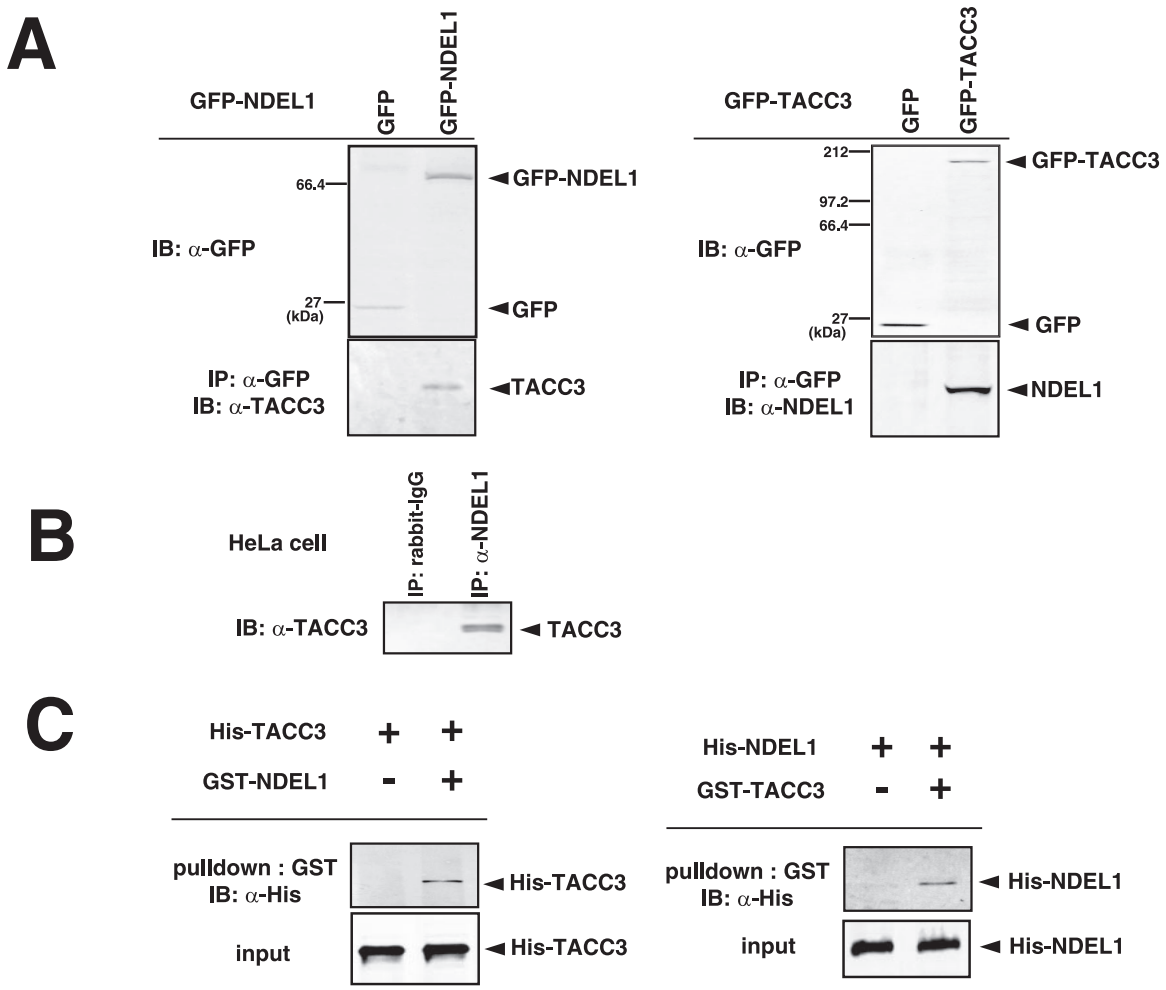


FIG. 4. Biochemical and genetic interaction between Aurora-A NDEL1 and TACC3. (A) Coimmunoprecipitation assays. HeLa cells were transfected with expression vectors as indicated. Immunoprecipitation (IP) was carried out using anti-GFP (α-GFP), and immunoblotting (IB) was performed with the anti-NDEL1 or anti-TACC3 antibody. Notice that the mobility of GFP-TACC3 is larger than the expected molecular mass from previous reports (19). (B) Coimmunoprecipitation assays using an anti-NDEL1 antibody. Endogenous NDEL1 clearly binds to TACC3. (C) GST pull-down. In vitro-translated HIS-NDEL1 and HIS-TACC3 were purified and incubated with purified GST-TACC3 and GST-NDEL, respectively, which were immobilized on glutathione-Sepharose 4B beads. Bound proteins were analyzed by Western blotting using an anti-HIS antibody. (D) Examination of phosphorylation and subcellular localization of NDEL1 and TACC3 in Aurora-A-depleted HeLa cells. Aurora-A-depleted HeLa cells exhibited dispersed distribution of NDEL1 and TACC3. The phosphorylation of both proteins was undetectable. (E) Examination of the phosphorylation and subcellular localization of Aurora-A and TACC3 in *Ndel1*-disrupted MEF cells. TACC3 appeared broadly distributed and lacked phosphorylation, whereas Aurora-A displayed phosphorylation at T288, suggesting that Aurora-A was activated despite the disruption of *Ndel1* and that NDEL1 triggers centrosomal targeting of TACC3 and phosphorylation. (F) Examination of phosphorylation and subcellular localization of Aurora-A and NDEL1 in TACC3-depleted HeLa cells. Phosphorylation and subcellular localization of Aurora-A and NDEL1 appeared grossly normal despite the depletion of TACC3. RNAi, RNA interference; −, absence of; +, presence of.

activation of Aurora-A and the phosphorylation of NDEL1 by Aurora-A preceded the phosphorylation of TACC3 by Aurora-A.

We compared Aurora-A-mediated S251 phosphorylation with CDK1-mediated T219 phosphorylation of NDEL1 (Fig. 2C). T219 phosphorylation began during prophase instead of the late G_2 prophase transition and continued throughout metaphase. To further clarify the temporal relationship between the enzymatic activity and S251 phosphorylation, we performed immunoblot analysis (Fig. 2D), which revealed that Aurora-A was activated at 8 h after the release of the double-thymidine block and continued to be active throughout metaphase. S251 phosphorylation appeared at 8 h after release but was downregulated soon afterwards, consistent with the immunohistochemistry data. In contrast, T219 phosphorylation appeared at 8.5 h after release and continued throughout mitosis. To address the mechanism of the sudden downregulation of S251, we examined S251 phosphorylation in the presence of MG132, which is a proteasome-specific inhibitor, or OA, which is a phosphatase inhibitor. MG132 significantly prolonged S251 phosphorylation. In contrast, OA was not effective in preventing the disappearance of S251 phosphorylation. These findings suggest that Ser251 phosphorylation may trigger proteasome-mediated rapid degradation and/or that the degradation cascade is activated during prophase (Fig. 2D).

Although Aurora-A was activated during mitosis, S251 phos-

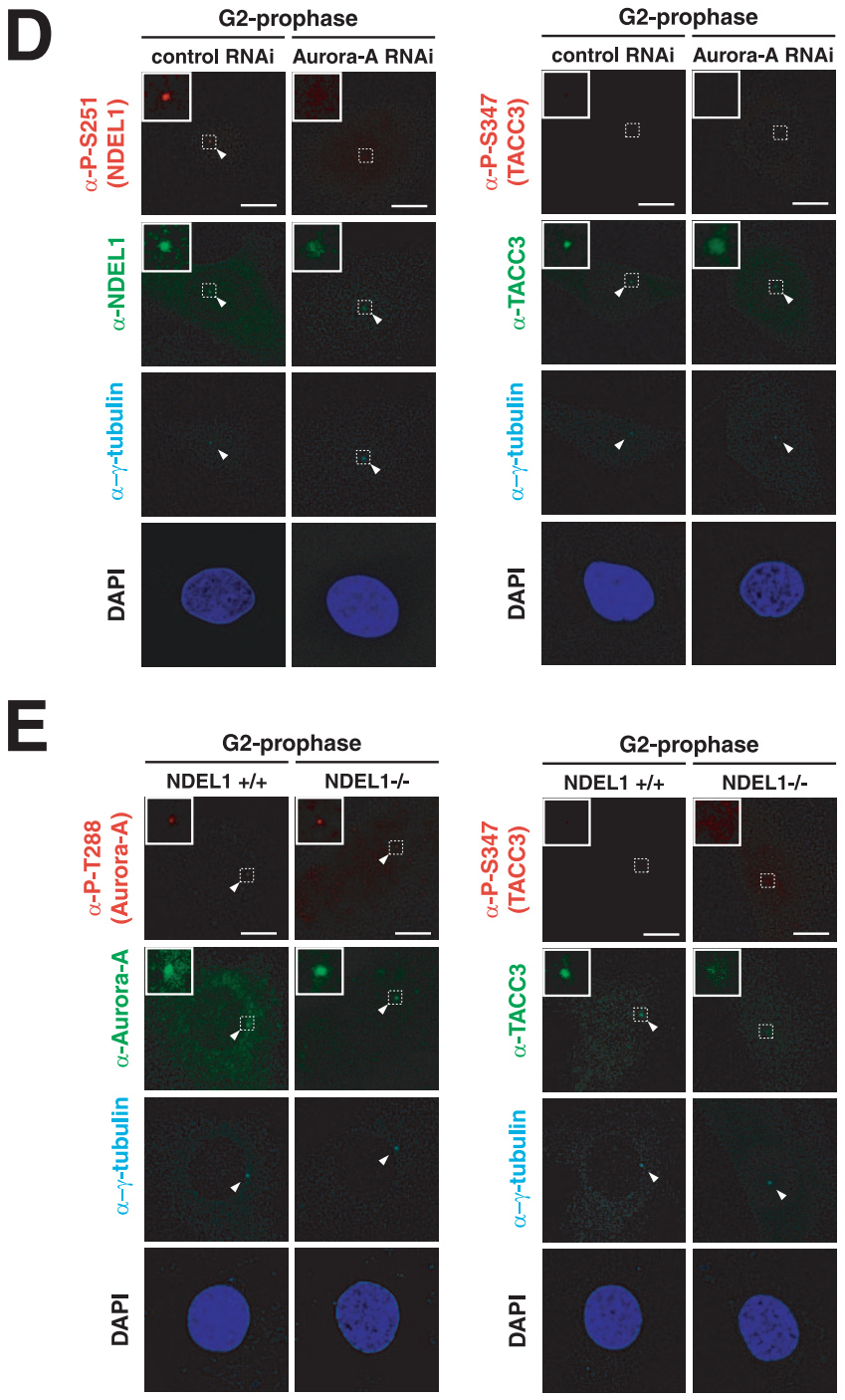


FIG. 4—*Continued.*

phorylation disappeared at the beginning of prophase, and the sudden downregulation of S251 NDEL1 was coincident with the phosphorylation of NDEL1 by CDK1. This suggests that the phosphorylation by CDK1 may inhibit further phosphorylation by Aurora-A. We therefore examined whether mutant NDEL1 in which phosphorylation sites by CDK1 were replaced by glutamic acid [GST-NDEL1(tripleS/T→E)] or alanine [GST-NDEL1(tripleS/T→A)] was a better or a worse substrate of Aurora-A. Compared to the wild-type NDEL1 or GST-NDEL1(tripleS/T→A), which was also a good substrate of Aurora-A, GST-NDEL1(tripleS/T→E) was a much less efficient substrate (Fig. 2E). In contrast, the replacement of S251 by glutamic acid [GST-NDEL1(S251E)] or alanine [GST-NDEL1(S251A)] did not have an obvious effect on the phosphorylation by CDK1 (Fig. 2F). As expected, mutation of the phosphorylation sites of Aurora-A or CDK1 resulted in the lack of phosphorylation of these sites (Fig. 2E and F). These findings suggest that NDEL1 is initially phosphorylated by

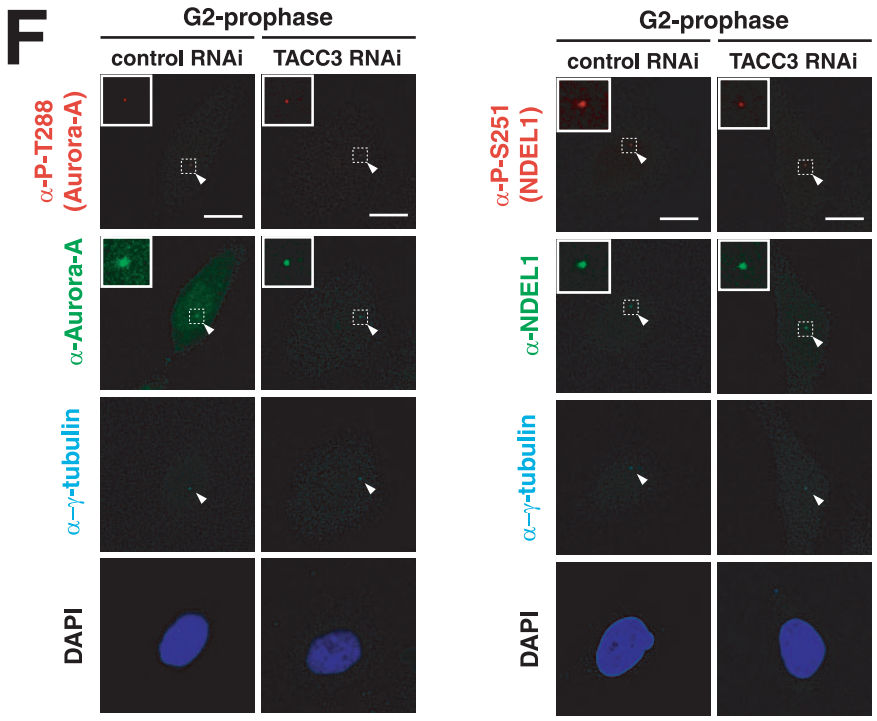


FIG. 4—*Continued.*

Aurora-A, followed by downregulation. Then, distinct pools of NDEL1 may be phosphorylated by CDK1.

Aurora-A-mediated phosphorylation of NDEL1 facilitates the centrosomal targeting of the phosphorylated NDEL1. To address the fate and subcellular localization of wild-type NDEL1 or phosphorylated NDEL1 in vivo, we generated MEF cells in which endogenous *Ndel1* was replaced by wild-type GFP-*Ndel1*s or mutated GFP-*Ndel1*s. Wild-type GFP-NDEL1 or mutated GFP-NDEL1s were expressed at fairly normal levels, and endogenous NDEL1 was not detected (see Fig. S3 in the supplemental material). MEF cells were synchronized and were examined at a given cell cycle phase. Wild-type GFP-NDEL1, GFP-NDEL1(S251E), appeared centrosomal at G_2 prophase compared to GFP-NDEL1(S251A), suggesting that S251 phosphorylation facilitated the limited distribution of NDEL1 to centrosomes (Fig. 3A). GFP-NDEL1(S251E) was stable during interphase, but it was more rapidly downregulated at prophase than GFP-NDEL1. This rapid decay was efficiently blocked by MG132 (Fig. 3A). These observations suggest that the phosphorylation of NDEL1 by Aurora-A triggers the ubiquitin-mediated protein degradation and/or that the degradation machinery is activated during prophase. Interestingly, the overexpression of GFP-NDEL1(S251E) resulted in the appearance of multiple centrosomes at prophase (Fig. 3A) (see below). GFP-NDEL1(tripleS/T→E) also displayed centrosomal localization that was less restricted at the centrosome (Fig. 3B). In addition, a signal was not detected by a phospho-S251-specific monoclonal antibody, which is consistent with biochemical data, suggesting that the phosphorylation by CDK1 may inhibit further phosphorylation by Aurora-A. Although GFP-NDEL1(tripleS/T→A;S251E) and GFP-NDEL1(tripleS/T→E;S251E) localized at centrosomes during prophase, GFP-NDEL1(tripleS/T→E;S251E) appeared to be less stable than other mutants (Fig. 3C). The treatment of MEF cells with MG132 improved the stability in either case, suggesting that the phosphorylation of S251 is critical for proteasome-mediated protein degradation (data not shown). While the expression of GFP-NDEL1(tripleS/T→E;S251E) does not have an obvious effect on interphase cells, expression during mitosis results in a cell with a multiple-centrosome pattern at prophase, suggesting that double phosphorylations by Aurora-A and CDK1 are not physiological and compromise centrosomal integrity. These multicentrosomal phenotypes associated with the expression of GFP-NDEL1(tripleS/T→E;S251E) were also observed in wild-type cells (Fig. 3D), suggesting that the effect of GFP-NDEL1(tripleS/T→E;S251E) expression is dominant.

We next documented the role of Aurora-A-mediated phosphorylation in the ubiquitination of NDEL1 (Fig. 3E). We extracted proteins from synchronized MEF cells, and this was followed by immunoprecipitation and immunoblotting. The ubiquitination of wild-type NDEL1 appeared at prophase, which was coincident with Aurora-A-mediated phosphorylation. In contrast, GFP-NDEL1(S251A) appeared to be more poorly ubiquitinated, whereas GFP-NDEL1(S251E) displayed higher ubiquitination (Fig. 3D), which was enhanced by MG132 (Fig. 3E). Our findings suggest that NDEL1 phosphorylation by Aurora-A triggers recruitment and restricted distribution of NDEL1 to the centrosome at G_2 prophase, and leads to rapid degradation by the ubiquitination-proteasome pathway. This order of the phosphorylation and the degradation appears to be critical for the maintenance of centrosome integrity since a multiple-centrosome pattern was observed after

the overexpression of GFP-NDEL1(S251E) or GFP-NDEL1 (tripleS/T→E;S251E).

Biochemical and genetic interactions between Aurora-A, NDEL1 and TACC3. Both NDEL1 and TACC3 are specific substrates of Aurora-A kinase (9, 19), and TACC3 is recruited to the centrosome in an Aurora-A-dependent fashion, suggesting that NDEL1 can also bind TACC3 directly. We first examined the physical interaction between NDEL1 and TACC3 by coimmunoprecipitation. HeLa cells were transfected with GFP-*Ndel1* or GFP-*Tacc3*. An anti-GFP antibody was used for immunoprecipitation, and an anti-TACC3 antibody and an anti-NDEL1 antibody were used for detection. GFP-NDEL1 was coimmunoprecipitated with endogenous TACC3, but not with GFP used as a negative control (Fig. 4A). Conversely, when GFP-TACC3 was immunoprecipitated with an anti-GFP antibody and blotted with an anti-NDEL1 antibody, similar results were achieved (Fig. 4A). The interaction of endogenous NDEL1 and TACC3 was confirmed by immunoprecipitation using an anti-NDEL1 antibody (Fig. 4B). These results suggested that NDEL1 specifically recognizes TACC3. The direct physical interaction between NDEL1 and TACC3 in vitro was verified by GST-pull-down analysis. HIS-TACC3 was pulled down by association with GST-NDEL1 (Fig. 4C), and HIS-NDEL1 was pulled down by association with GST-TACC3 (Fig. 4C). No interaction was detected between the GST control and HIS-NDEL1 or HIS-TACC3. These results further suggested that NDEL1 bound directly with TACC3. We quantitated the affinity between NDEL1 and TACC3 by Biacore using recombinant proteins (see Fig. S4A in the supplemental material). Specific interaction between NDEL1 and TACC3 was detected at an extraordinarily strong affinity [K_A (1/M), $9.45 \times 10^9 \pm 3.52 \times 10^9$], an effect that was completely reproduced when the protein combinations were reversed [K_A (1/M), $1.33 \times 10^{10} \pm 0.34 \times 10^{10}$] (see Fig. S4B in the supplemental material). Wild-type NDEL1 and NDEL1 mutated at sites of phosphorylation by Aurora-A and CDK1 displayed similar binding affinities to TACC3, suggesting that the phosphorylation of NDEL1 does not influence the binding between NDEL1 and TACC3 [K_A (1/M), $9.82 \times 10^9 \pm 3.92 \times 10^9$, GST-NDEL1(S251A)] [$K_A$ (1/M), $8.98 \times 10^9 \pm 4.01 \times 10^9$, GST-NDEL1(S251E)] (see Fig. S4C in the supplemental material).

To characterize how these three proteins are related, we examined whether the inactivation of each protein influences the localization and the phosphorylation of other proteins. To evaluate the role of Aurora-A in the phosphorylation and distribution of target proteins, we used siRNA to reduce the levels of Aurora-A in HeLa cells (16, 23) and examined the phosphorylation of each protein and the centrosome morphology. In the series of experiments, we defined early prophase as the beginning of chromosome condensation with a single centrosome and prophase as the completion of chromosome condensation with separated centrosomes. Aurora-A depletion by siRNA significantly reduced the phosphorylation level and centrosomal accumulation of NDEL1 and TACC3, as in the cases of lower organisms (12) (Fig. 4D). We previously generated conditional knockout mice of *Ndel1* by CRE-mediated homologous recombination (33). Therefore, we disrupted *Ndel1* by the expression of the *Cre* gene in established MEF cells. *Ndel1*-disrupted MEF cells displayed various impairments of cell cycle progression, including centrosome maturation defects, separation defects, and spindle aberrations, such as weak or split spindles (data not shown). T288 phosphorylation of Aurora-A was normal, suggesting that the activation of Aurora-A does not require the presence of NDEL1. However, centrosomal targeting and S347 phosphorylation of TACC3 were severely impaired despite the normal activation of Aurora-A (Fig. 4E). Finally, we knocked down TACC3 expression by siRNA (19). The reduction of TACC3 levels resulted in misaligned chromosomes that are indicative of the TACC3 RNA interference phenotype as previously reported (19). Most interestingly, TACC3 depletion resulted in no gross abnormalities in the phosphorylation of Aurora-A or NDEL1 and normal centrosomal targeting of NDEL1 (Fig. 4F), suggesting that TACC3 likely does not substantially contribute to the activation of Aurora-A and NDEL1 at mitotic entry. Our findings suggest that NDEL1 is located at the middle of the pathway between Aurora-A and TACC3.

Phosphorylation-mimetic mutants of NDEL1 rescued the mitotic entry defect by Aurora-A depletion. Aurora-A has been implicated in the regulation of centrosome maturation and spindle formation (1, 15, 36). We demonstrated that NDEL1 is a substrate of Aurora-A and is specifically phosphorylated at mitotic entry. We also found that NDEL1 is required for TACC3 targeting to the centrosome. These observations suggest that the phosphorylation of NDEL1 by Aurora-A might be essential for mitotic entry. To address the role of S251 phosphorylation, we examined whether the expression of *Ndel1* or mutant forms of *Ndel1* rescues the phenotypes generated by siRNAs against Aurora-A. First, we examined the mitotic index. A significant reduction of the mitotic index suggests that the depletion of Aurora-A markedly impaired the ability of cells to enter mitosis (16). The expression of GFP-NDEL1(S251E), GFP-NDEL1(tripleS/T→A;S251E), and GFP-NDEL1(tripleS/T→E;S251E) in Aurora-A-depleted cells restored mitotic entry, whereas Aurora-A-depleted cells expressing other constructs failed to recover (Fig. 5A). These data suggest that the phosphorylation of NDEL1 by Aurora-A supports mitotic entry. γ-Tubulin is an indicator of centrosome maturation, which is required for MT nucleation (16, 23). We explored whether γ-tubulin accumulation was recovered by the expression of GFP-*Ndel1*s by the examination of intensity of immunocytochemistry (Fig. 5B). A reduction of the γ-tubulin signal by Aurora-A depletion was fairly well restored by the expression of GFP-NDEL1(S251E), GFP-NDEL1(tripleS/T→A;S251E), and GFP-NDEL1(tripleS/T→E;S251E), whereas cells expressing other constructs failed to recover the accumulation of γ-tubulin (Fig. 5B). We also examined whether the expression of NDEL1 and mutated NDEL1s can rescue a lack of centrosome targeting of TACC3 after the depletion of Aurora-A by siRNA. Importantly, the recovery of centrosomal targeting of TACC3 by the expression of GFP-NDEL1(S251E), but not by GFP-NDEL1(S251A) (Fig. 5C), was observed. These findings suggest that NDEL1 is a key molecule for the recruitment of proteins to the centrosome that are downstream of Aurora-A.

Centrosome separation and chromosome condensation are important indicators of the normal progression of prophase. These two events are independently but coordinately regulated. Aurora-A depletion by siRNA leads to separation defects of centrosomes, whereas chromosome condensation is

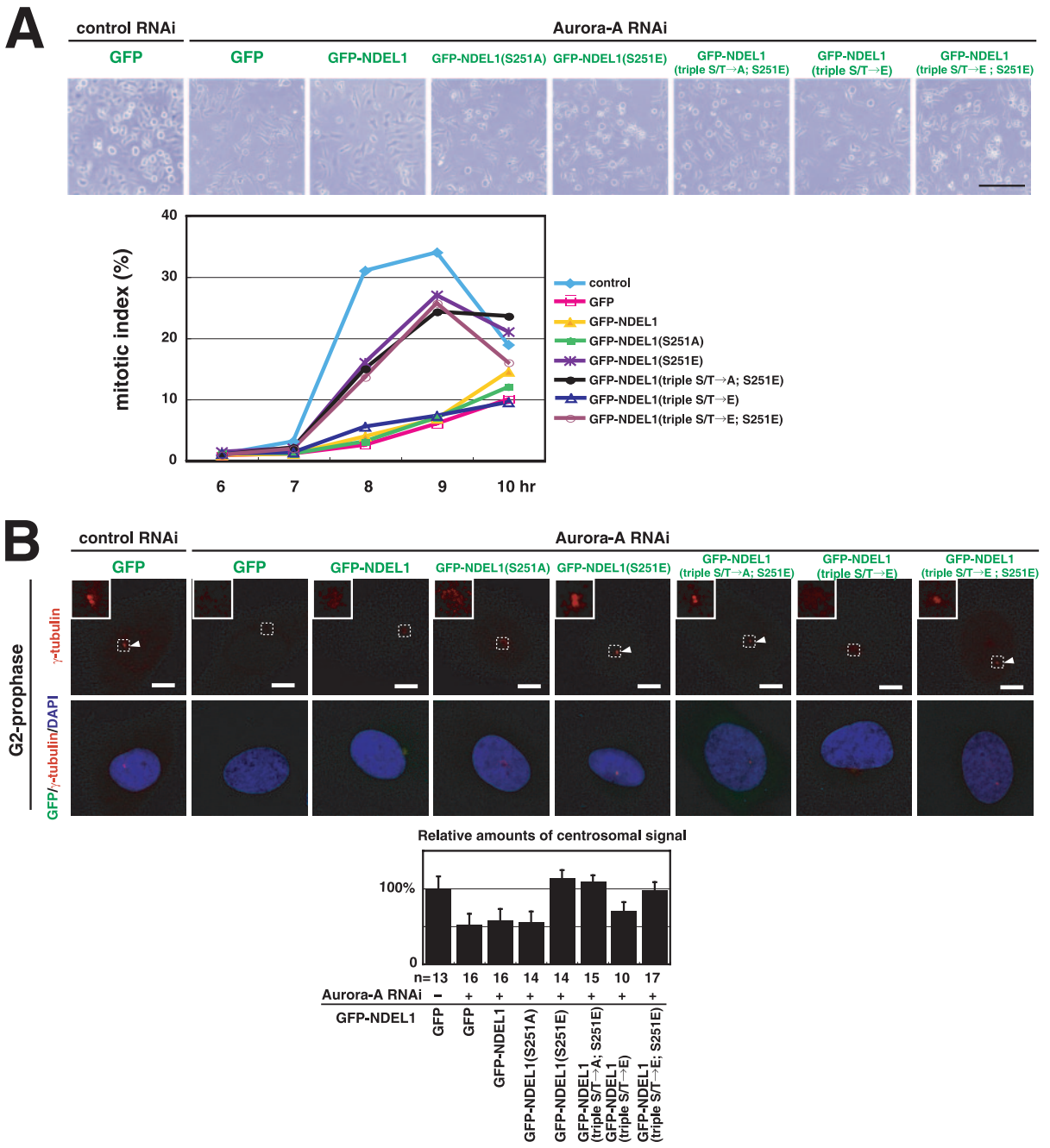


FIG. 5. Phosphorylation-mimetic NDEL1 mutant rescued impairment of separation and maturation defect of the centrosome by Aurora-A depletion. (A) To examine whether the expression of phosphorylation-mimetic mutants of NDEL1 support mitotic entry of the HeLa cells in which Aurora-A is depleted by siRNA, we measured the mitotic index of synchronized HeLa cells. Phase-contrast images of cells transfected with control GFP and GFP-*Ndel1* constructs and a time course of the mitotic index of cells transfected (shown at the bottom). (B) γ-Tubulin is an essential component for MT nucleation. Centrosomes mature as cells enter mitosis, accumulating γ-tubulin. This occurs concomitantly with an increase in the number of centrosomally organized MTs. We examined the recovery of γ-Tubulin accumulation by expression of mutated NDEL1s under Aurora-A depletion. γ-Tubulin accumulation was estimated by the distribution and intensity of the immunocytochemistry, as indicated. The expression of GFP-NDEL1(S251E), GFP-NDEL1(tripleS/T→A;S251E), and GFP-NDEL1(tripleS/T→E;S251E) improved γ-tubulin accumulation to the centrosome, whereas control GFP, GFP-NDEL1, GFP-NDEL1(S251A), and GFP-NDEL1(tripleS/T→E) did not. The accumulation of γ-tubulin was quantitated by measuring the relative fluorescence intensity of 200-nm circles around the centrosome. Error bars indicate standard deviations. (C) Examination of subcellular localization of TACC3 in Aurora-A-depleted HeLa cells by the expression of mutant *Ndel1*s. Aurora-A depletion resulted in dispersed localization of TACC3. Exogenous expression of GFP-NDEL1(S251E) restored the centrosomal distribution of TACC3 as indicated. In contrast, the expression of wild-type GFP-NDEL1 or GFP-NDEL1(S251A) failed to restore the impairment of TACC3 localization. (D) At prophase, the separation of centrosomes commenced and moved to opposite poles, associated with a maturation step. HeLa cells that lack Aurora-A displayed an impairment of separation and an attached appearance. The impairment of separation of the centrosomes was efficiently and partially rescued by GFP-NDEL1(S251E) and GFP-NDEL1(tripleS/T→A;S251E), respectively. Control GFP, GFP-NDEL1, GFP-NDEL1(S251A), and GFP-NDEL1(tripleS/T→E) were less efficient in rescuing this phenotype. Note the separation of multiple centrosomes after the transfection of GFP-NDEL1(tripleS/T→E;S251E). Quantitation was carried out by measuring the distance between two centrosomes as shown at the bottom. Error bars indicate standard deviations. RNAi, RNA interference; −, without; +, with.

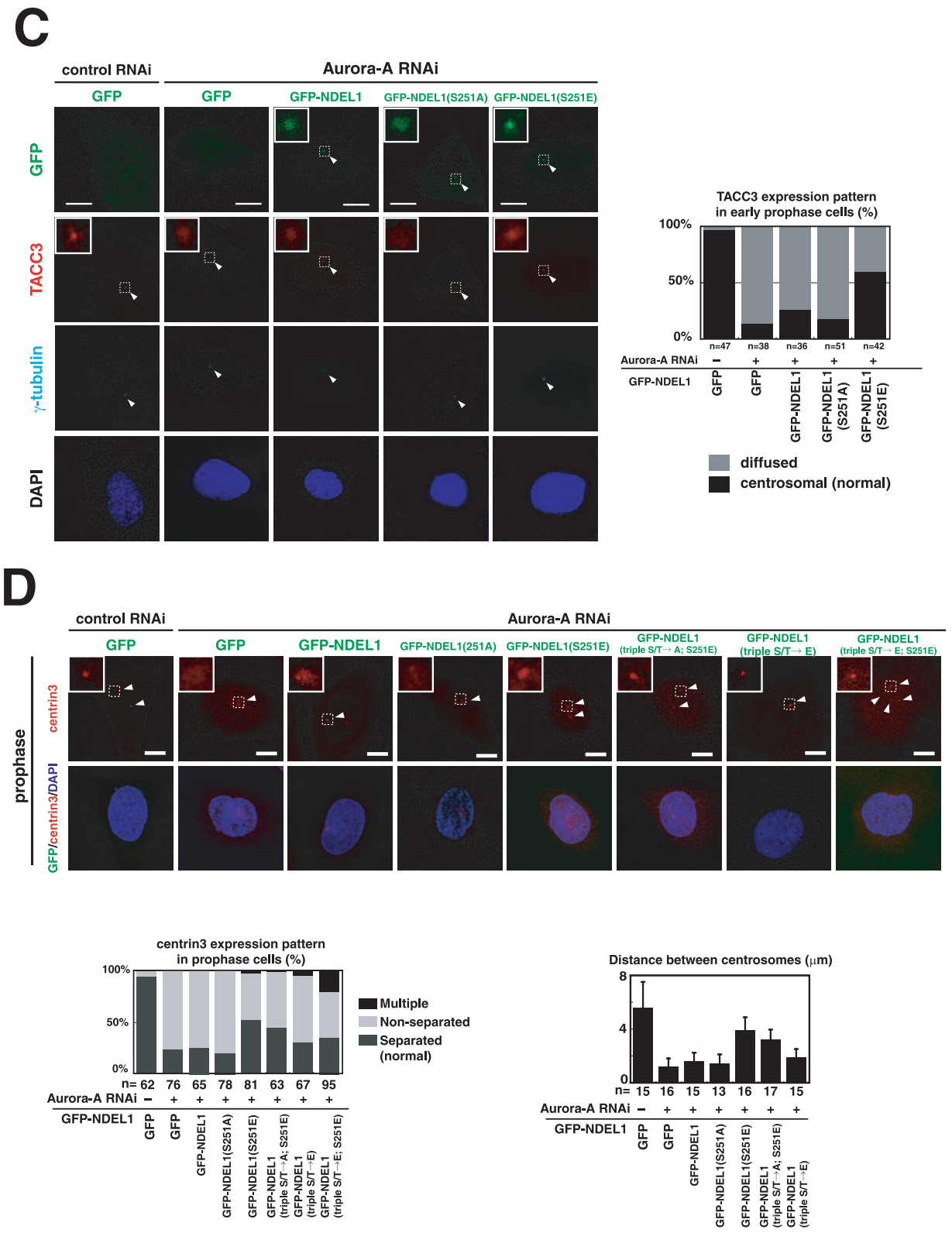


FIG. 5—*Continued.*

maintained fairly intact (16, 23). We selected HeLa cells with chromosome condensation as prophase HeLa cells and explored whether centrosome separation was restored by the expression of phosphorylation-mimetic NDEL1s. Centrosome separation during prophase was observed after the transfection of GFP-NDEL1(S251E) and GFP-NDEL1(tripleS/T→A; S251E). We measured the distance between two centrosomes at prophase by using an anti-centrin-3 antibody as a centriole

marker (16). The results of quantitation revealed that the transfection of GFP-NDEL1(S251E) and GFP-NDEL1 (tripleS/T→A;S251E) resulted in separations of 3.9 μm ± 1.1 μM and 3.3 μm ± 0.9 μM, respectively (Fig. 5D), suggesting that the expression of GFP-NDEL1(S251E) leads to more normal centrosomal separation. In contrast, clear centrosome separation was not observed after the expression of GFP, GFP-NDEL1, GFP-NDEL1(S251A), and GFP-NDEL1(tripleS/T→E). These findings indicate that the phosphorylation of NDEL1 is essential to support centrosome maturation and separation during mitosis.

DISCUSSION

NDEL1 is a binding partner of LIS1, and this interaction regulates dynein motor function and katanin localization (8, 26, 32, 38). In particular, NDEL1 is phosphorylated by CDK1, and this phosphorylation is essential for the proper targeting of NDEL1 binding proteins to the centrosome (38). We have demonstrated that NDEL is also a target of Aurora-A kinase. The phosphorylation of NDEL1 by Aurora-A enhanced the affinity of two proteins and results in the more restricted distribution of NDEL1 at the centrosome. Interestingly, NDEL1 is differentially phosphorylated by Aurora-A and CDK1. It is possible that distinct pools of NDEL1 may be targeted by each kinase, or conversely, the effects of each kinase may counteract each other within the same pool of NDEL1. In vitro kinase assays also revealed that CDK1-mediated phosphorylation of NDEL1 prevents further phosphorylation by Aurora-A, thereby avoiding double phosphorylation. Persistent and simultaneous phosphorylation of NDEL1 by both Aurora-A and CDK1 results in the fragmentation of the centrosome, suggesting that ordered phosphorylation by Aurora-A and CDK1 is required for the maintenance of centrosomal integrity during mitosis.

Aurora-A is essential for centrosome separation and maturation, as well as the creation of the spindle body (2, 11, 13). We found that NDEL1 displayed high affinity for TACC3, a target of Aurora-A. We also found that *Ndel1* disruption severely impaired centrosomal targeting of TACC3. These observations suggest that NDEL1 mediates protein recruitments to the centrosome at mitotic entry. This first cohort of protein recruitment driven by Aurora-A to the centrosome may be essential for centrosome maturation and initiation of spindle formation. After this is accomplished, it appears that NDEL1 is phosphorylated by CDK1, which promotes the recruitment of katanin p60 to facilitate MT remodeling. The overexpression of GFP-NDEL1(tripleS/T→E) results in weaker spindles (our unpublished observations), which supports our interpretation. These sequential events might be essential for spindle formation through the proper remodeling of microtubules.

Centrosomes (and other microtubule-organizing centers) are made up of numerous proteins whose amino acid sequences suggest a coiled-coil tertiary structure (7). Increasing evidence indicates that this molecular structure may be well designed for the organization of multiprotein scaffolds that can anchor a diversity of activities ranging from protein complexes involved in MT nucleation to multicomponent pathways for cellular regulation (7). By physically linking components of a common pathway, molecular scaffolds can increase the local concentration of components, limit nonspecific interactions, and provide spatial control for regulatory pathways by positioning them at specific sites in the proximity to downstream targets or upstream modulators. Our preliminary biochemical analysis suggested that Aurora-A-mediated phosphorylation of NDEL1 leads to structural changes and exposes additional protein binding sites, which could be employed to create larger NDEL1 oligomers/polymers (our unpublished observations). This structural change may be essential for the recruitment and sequestration of NDEL1 at the centrosome. The recruitment of NDEL1 may further promote the centrosomal targeting of other proteins, including LIS1, katanin p60, and cytoplasmic dynein heavy chain. These phenomena appear to provide positive feedback to achieve the recruitment of many components at the centrosome during the short time frame of mitosis. Interestingly, Aurora-A-mediated phosphorylation of NDEL1 was rapidly downregulated at the prophase-metaphase transition by ubiquitination-mediated degradation. We found that NDEL1 forms a very stable protein complex with TACC3, with a very small dissociation constant. Interestingly, NDEL1 prohibits the phosphorylation of TACC3 by Aurora-A and subsequent binding to CH-TOG/XMAP215 (10, 22, 37), which implies that NDEL1 must be removed from TACC3 for the further progression of mitotic events (our unpublished observations). Proteasome-dependent protein degradation might be important for the coordination of proper recruitment and progression of the cell cycle. The proper regulation of the phosphorylation and accumulation of NDEL1 at the centrosome may provide a scaffold for the recruitment of centrosomal proteins, which are essential for the maturation and separation of the centrosome. These recruitments of proteins are also essential for coordinated stabilization or remodeling of MTs and spindle formation at prometaphase.

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