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Id1 Overexpression Induces Tetraploidization and Multiple Abnormal Mitotic Phenotypes by Modulating Aurora A

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The basic helix-loop-helix transcription factor, Id1, was shown to induce tetraploidy in telomerase-immortalized nasopharyngeal epithelial cells in this study. Using both transient and stable Id1-expressing cell models, multiple mitotic aberrations were detected, including centrosome amplification, binucleation, spindle defects, and microtubule perturbation. Many of these abnormal phenotypes have previously been reported in cells overexpressing Aurora A. Further experiments showed that Id1 could stabilize Aurora A, whereas knocking down Aurora A expression in Id1-expressing cells could rescue some of the mitotic defects. The mechanisms by which Aurora A could be modulated by Id1 were explored. DNA amplification of the Aurora A locus was not involved. Id1 could only weakly activate the transcriptional activity of the Aurora A promoter. We found that Id1 overexpression could affect Aurora A degradation, leading to its stabilization. Aurora A is normally degraded from mitosis exit by the APC/C^{Cdh1}-mediated proteasomal proteolysis pathway. Our results revealed that Id1 and Cdh1 are binding partners. The association of Id1 and Cdh1 was found to be dependent on the canonical destruction box motif of Id1, the increased binding of which may compete with the interaction between Cdh1 and Aurora A, leading to stabilization of Aurora A in Id1-overexpressing cells.

INTRODUCTION

Id1 is a dominant-negative inhibitor of the basic helix-loop-helix (bHLH) transcription factors. The Id1 protein lacks the basic DNA binding domain and thus antagonizes the transcriptional activity of many differentiation-specific bHLH transcription factors by forming DNA binding-incompetent heterodimers (Benezra *et al*., 1990). It has been reported that Id1 could also interact with non-bHLH transcription factors (Roberts *et al*., 2001) to interfere the Rb regulatory pathway (Hara *et al*., 1996). Id1 suppresses cellular senescence (Ohtani *et al*., 2001), and it facilitates immortalization by repressing p16^{INK4A} in human fibroblasts (Alani *et al*., 2001). Overexpression of Id1 is common in immortalized cells that have escaped cellular senescence (Hara *et al*., 1994). In our previous study, Id1 expression was expressed in nasopharyngeal epithelial cells before and after immortalization but not in senescent cells (Li *et al*., 2006). Id1 overexpression has been implicated in multiple human cancers, including nasopharyngeal carcinoma (Wang *et al*., 2002). The role of Id1 in tumor neo-angiogenesis and cancer metastasis has been addressed previously (Ruzinova *et al*., 2003; Minn *et al*., 2005). Therefore, Id1 is being marked as an attractive target for anticancer therapy. We have previously reported that the Epstein–Bar virus encoded latent membrane protein 1 is able to up-regulate Id1 expression in immortalized nasopharyngeal epithelial cells (Li *et al*., 2004). Apart from the involvement of Id1 in facilitating cell immortalization, Id1 is also implicated in mitotic regulation. Id1 could be localized to the centrosomes (Hasskarl *et al*., 2004), and ectopic overexpression of Id1 could rapidly induce supernumerary centrosomes in human foreskin keratinocytes, suggesting that Id1 could potentially contribute to carcinogenesis by centrosome amplification. Recently, the same group has reported that the ability of Id1 to induce supernumerary centrosomes may correlate with the interaction of Id1 with the proteosomal subunit S5A/Rpn10 (Anand *et al*., 1997; Hasskarl *et al*., 2007). In addition, Id2 contains a destruction-box (D-box) recognition sequence for interacting with the anaphase-promoting complex (APC/C) (Lasorella *et al*., 2006). The canonical D-box motif is commonly present in Id proteins, including Id1, suggesting that Id1 may also affect mitosis by interfering with the APC/C.

Aurora A is a centrosome-localized mitotic kinase involved in assembly and stability of mitotic spindle. The *Aurora A* gene is located at chromosome position 20q13, a site frequently amplified in breast and colorectal tumors (Bischoff *et al*., 1998; Zhou *et al*., 1998; Bischoff and Plowman, 1999). Overexpression of Aurora A has been reported in many tumor cells (Miyoshi *et al*., 2001; Goepfert *et al*., 2002; Tatsuka *et al*., 2005). In vitro studies revealed multiple mitotic abnormalities after Aurora A overexpression, including nonfunctional bipolar spindle (Marumoto *et al*., 2003), cytokinesis failure, polyploidy (Meraldi *et al*., 2002), and centrosome amplification (Miyoshi *et al*., 2001; Marumoto *et al*., 2003). Immortalized cells expressing the inactive form of Aurora A could also result in tetraploidization (Anand *et al*., 2003; Marumoto *et al*., 2003). That overexpression of active or inactive Aurora A

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always provokes aneuploidy and centrosome amplification (Goepfert *et al.*, 2002; Marumoto *et al.*, 2003) suggests that Aurora A is crucial for the induction of chromosome instability; hence, it plays an important role in the early steps of tumorigenesis. Despite the difficulties with chromosome alignment, Aurora A-overexpressing cells do exit mitosis (Anand *et al.*, 2003), but they may result in cytokinesis defects. Cells lacking functional p53 may continue to cycle to form polyploid intermediates and eventually achieve aneuploidy with amplified centrosomes (Meraldi *et al.*, 2002). In somatic cells, Aurora A levels are low during G1 and S phase, peak during G2 and mitosis, and drop during mitotic exit into G1 phase of the next cell cycle (Bischoff and Plowman, 1999). The protein is degraded during mitotic exit by the APC/C^{Cdh1}, which requires Cdh1 as the coactivator. When Cdh1 recognizes and binds to its substrates, including Aurora A, the ubiquitin reaction will be activated, leading to proteasomal degradation of the substrate (Crane *et al.*, 2004; Pfleger *et al.*, 2001; Taguchi *et al.*, 2002; Walter *et al.*, 2000; Zachariae, 2004).

In this study, we reported that Id1 overexpression induces multiple mitotic aberrations, many of which are mediated through Aurora A. We demonstrate that Id1 could interact with the anaphase-promoting complex coactivator, Cdh1, to stabilize Aurora A. The binding ability between Id1 and Cdh1 involves the Id1 D-box, which could be recognized by the APC/C^{Cdh1}. Our study has revealed some of the mechanisms involved Id1 induction of abnormal mitotic phenotypes in immortalized human epithelial cells and cancer cells.

MATERIALS AND METHODS

Establishment of Immortalized Nasopharyngeal Epithelial Cell Line with Stable Expression of Id1 and Other Cell Lines Used

The nasopharyngeal immortalized cell line NP460hTert was established previously in our laboratory (Li *et al.*, 2006). The immortalized cells were maintained in a 1:1 ratio of defined keratinocyte-SFM (Invitrogen, Carlsbad, CA) and EpiLife medium supplemented with growth factors (Sigma-Aldrich, St. Louis, MO) at 37°C with 5% CO_2. High level of expression of Id1 was induced by retroviral transduction of Id1 into NP460hTert at population doubling 62. The pcDNA expression plasmid of Id1 was a gift from Dr. E. Hara (Paterson institute for Cancer research, Manchester, United Kingdom), which contains the full-length human Id1 gene (Hara *et al.*, 1994). The full-length Id1 gene was subcloned into a retrovirus construct (pBabe-puro) as described previously (Ling *et al.*, 2003). The construct, containing the retroviral vector, was then transfected into PT67 packaging cells (BD Biosciences, Palo Alto, CA) to produce infectious virus according to the manufacturer's protocol. The retroviral supernatants were filtered and stored at −70°C until use. NP460 hTert cells, plated at 1×10^5 cells/T_{25} flask, were transduced by adding the retroviral supernatant plus 4 μg/ml Polybrene (Sigma-Aldrich) for 6 h. After infection, cells were selected with puromycin at 0.8 μg/ml for a period of 14 d. Stable expression of Id1 in the NP460hTert-transduced cells (NP460hTert-Id1) was confirmed by Western blotting. The HeLa cell line was obtained from the American Type Culture Collection (Manassas, VA). The HONE1 cell line was kindly provided by Dr. Ron Glaser (The Ohio State University) (Yao *et al.*, 1990). Both the HeLa and HONE1 cells were maintained in RMPI 1640 medium supplemented with 10% fetal calf serum (FCS) (1% penicillin/streptomycin), and they were incubated at 37°C with 5% CO_2. The U2OS (osteosarcoma cell line) was from American Type Culture Collection. U2OS cells were maintained in DMEM supplemented with 10% FCS (1% penicillin/streptomycin).

Transient Transfections and Construction of Id1 D-Box Mutants

Transient transfections were achieved using either FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) or Lipofectamine 2000 (Invitrogen) according to the manufacturers' recommendations. Forty-eight hours after transfection, cells were either collected for immunoprecipitation studies, Western blotting analysis, or examined by immunofluorescence analysis. The expression plasmids pDSRed (transfection marker) and pEGFP-α-tubulin (microtubule marker) were obtained from Clonetech (Mountain View, CA) (Rosa *et al.*, 2006). The pcDNA-flag-Id1wt, pcDNA-ΔN-flag, pcDNA-ΔC-flag was kindly provided by Dr. J. Hasskarl (Department of internal medicine, University of Freiburg, Freiburg, Germany) (Hasskarl *et al.*, 2004). The Id1 D box mutants pcDNA-flag-Id1mDB_{126}, pcDNA-flag-Id1mDB_{129}, and pcDNA-flag-Id1mDB_{133} were generated by QuikChange site-directed mutagenesis kit from the pcDNA-flag-Id1wt plasmid (Stratagene, La Jolla, CA). Mutagenic primers were designed using the Web-based QuikChange primer design program. All sequences were verified by DNA sequencing.

Gene Silencing by Short Hairpin RNA (shRNA) and Dual-Luciferase Analysis

The short hairpin RNA targeted specifically against Aurora kinase A (pSR-shAURKA) and the empty vector (pSR-shGL2) were kindly provided by Prof. Horii Akira (Tohoku University, Sendai Miyagi, Japan) (Hata *et al.*, 2005). The antisense expression plasmid pcDNA3-ASId1 was used as described previously (Ling *et al.*, 2003). Gene silencing was achieved by transient transfection into cell lines with high level of Aurora kinase A by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Dual-luciferase analysis was performed as described previously (Man *et al.*, 2007). Cells (1×10^6) were transiently cotransfected with 0.5 μg of pcDNA3-Id1 plasmid DNA and 0.5 μg of different 5′-deletion Aurora A promoter-luciferase constructs (pGL1486, pGL189, pGL124, pGL75, and pGLbasic). The promoter luciferase constructs were provided by Prof. Yoshiaki Ishigatsubo (First Department of Internal Medicine, Tokyo Medical and Dental University, Tokyo, Japan) (Tanaka *et al.*, 2002). To allow normalization of transfection efficiencies between different plates, each transfection also contains 0.01 μg of promoter-driven *Renilla* luciferase reporter plasmid (pRL-conA). Constant amount of DNA per transfection was ensured. Luciferase assay was performed according to the manufacturer's instructions (Dual-Luciferase Reporter kit, Promega, Madison, WI). Measurements of each data point were taken on triplicate wells. The readings were normalized with the *Renilla* luciferase activities. Three separate experiments were performed and the error bars represent the SE of the means.

Cytogenetic Analysis and Fluorescent In Situ Hybridization (FISH)

A complete cytogenetic profile of NP460 hTert and NP460 hTert-Id1 cells at comparable population doublings were analyzed by conventional cytogenetics method as described previously (Man *et al.*, 2007). Thirty cells from different passages of each cell line were karyotyped according to the International System for Human Cytogenetic Nomenclature (ISCN 1995) using the Cytovision software (Applied Imaging, San Jose, CA).

The PAC clone (RP5-843L14), located at chromosome 20q13.2 in which *Aurora A* resides was obtained from Invitrogen (Chung *et al.*, 2005). Another PAC clone (RP5-1107C24), juxtaposed to RP5-843L14 labeled with spectrum green, was used as an internal control. For each labeling, 0.5 μg genomic DNA was used. Random Primer Mix (Bioprime kit; Invitrogen) was added to the DNA. The DNA/primer mix was denatured at 100°C and quenched on ice. The DNA-labeling reaction was performed by combining DNA/Random Primer mix, 10× dNTP (1.2 mM dATP, 1.2 mM dGTP, 1.2 mM dCTP, and 0.6 mM dTTP), Spectrum Red-dUTP (Vysis, Downer's Grove, IL), 10× reaction buffer, nuclease-free water and Klenow enzyme (50 U/μl). The reaction was incubated at 37°C overnight, and purified by the Quick Spin Sephadex G-50 spin column (GE Healthcare, Chalfont St. Giles, United Kingdom). The collected flow through was precipitated with 3 M sodium acetate and cold ethanol overnight. Labeled PAC clone was rehydrated with hybridization buffer (Vysis) and human cot-1 DNA (Invitrogen). Chromosome spreads were prepared as described previously (Man *et al.*, 2007). The chromosomes were denatured with 70% formamide/30% 2× SSC at 70°C. The hybridization, washing, and image capturing procedures were performed as described previously (Chung *et al.*, 2005; Li *et al.*, 2006).

Western Blotting and Immunoprecipitation Studies

A detailed protocol for Western blotting was described previously (Li *et al.*, 2006; Man *et al.*, 2007). In this study, primary antibodies used and the optimal concentration used for 25 μg of protein lysates were Id1 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), Aurora A (1:400; Santa Cruz Biotechnology), cdh1 (1:500; Zymed Laboratories, South San Francisco, CA), Flag monoclonal M2 (1:3000; Sigma-Aldrich), Plk1 (1:500; Bethyl Laboratories, Montgomery, TX), Survivin (1:200; Zymed Laboratories), Aurora B (1:1000; Abcam, Cambridge, United Kingdom), and β-actin (1:1000; Santa Cruz Biotechnology, CA). Expression of actin was used as loading control. For antibodies immunoblotted against nuclear proteins, histone was used as a loading control. Relative protein expression levels were quantified by comparing the density of the bands by using a gel documentation system (Ultraviolet Products, San Gabriel, CA). Where indicated, 5 μg/ml cylcloheximide was used to inhibit protein synthesis; and 1 μM MG132 (Calbiochem, San Diego, CA) was used for preventing proteasomal-mediated proteolysis. For coimmunoprecipitation studies on the interaction between Id1 and Aurora A/cdh1, 150 μg of lysates (transfected with either empty vector or Id1) were prepared: flag, Aurora A, or cdh1 immunoprecipitates were recovered on protein A/G agarose beads (Calbiochem, San Diego, CA) for 4 h at 4°C. The immunoprecipitated complexes with sample buffer were subjected to electrophoresis on a 12.5% SDS-polyacrylamide gel and immunoblotted with the specific antibodies.

Immunofluorescence Staining

Cells grown on coverslips were fixed with methanol at −20°C. Conventional immunofluorescence staining technique was performed as described previously (Man *et al*., 2007), with particular reference for immunofluorescent staining of microtubule markers (Rosa *et al*., 2006). Antibodies used for immunofluorescence staining included the following: murine monoclonal anti-γ-tubulin (Sigma-Aldrich), rabbit anti-centrin (Sigma-Aldrich), anti-rabbit-Id1 (Santa Cruz Biotechnology), anti-goat Aurora kinase-A (Santa Cruz Biotechnology, CA), anti-mouse-DM1α (antibody against α-tubulin) (Gromley *et al*., 2003), anti-mouse monoclonal Flag M2 (Sigma-Aldrich), Alexa Fluor 488- or Alexa Fluor 568-conjugated secondary antibodies (Invitrogen) and fluorescein isothiocyanate (FITC)-conjugated secondary antibody. Nuclei were visualized with 4,6-diamidino-2-phenylindole (Vysis). For centrosome scoring, at least 200 cells were evaluated in each cell line. Cells with three or more γ-tubulin signals were considered abnormal. For mitotic spindle examination, 50 mitotic cells were examined in each cell line, and cells that lack bipolar spindles were considered abnormal.

Cell Cycle Distribution Analysis, Cell Synchronization, and Bivariate Antigen-DNA Flow Analysis

Cell cycle analysis was performed to confirm ploidy change on a flow cytometer EPICS profile analyzer and analyzed using the ModFit LT2.0 software (Beckman Coulter, Fullerton, CA). The cells (1×10^6) were fixed with cold 70% ethanol. Fixed cells were treated with DNase-free RNase followed by 100 μg/ml propidium iodide staining to reveal the DNA content.

To obtain cells primarily in G1 phase and S phase, asynchronous cells were treated with 6 μM aphidicolin (Sigma-Aldrich) for 19 h. Cells were released by washing twice in serum-free medium. For late G1 phase, cells were reincubated in serum-containing medium for an additional 6 h before collection and an additional 8 h for S phase collection. HeLa cells were synchronized in G2/M phase by 0.3 μM nocodazole block for 12 h for immortalized cells and for 15 h for HeLa cells. The efficacy of the synchronization was confirmed by fluorescence-activated cell sorting (FACS).

Cells were fixed and washed with phosphate-buffered saline (PBS) supplemented with 2% FCS and 0.1% sodium azide. Resuspended cells were permeablized in 0.25% Triton X-100 for 10 min at 4°C and subjected to primary antibody (1:40 dilution) overnight at 4°C. After washing, cells were incubated with secondary antibody conjugated with FITC (1:100). Cellular DNA was stained with propidium iodide (PI). Bivariate flow cytometric analysis was performed by quantification of FITC and PI fluorescence by using 514-nm bandpass filter and 600-nm bandpass filter, respectively. Data were interpreted using the EXPO2 flow cytometer analysis software (Beckman Coulter).

Live Cell Imaging and Time-Lapse Microscopy

Cells were seeded onto glass bottom microwell dishes (MatTek, Ashland, MA) or 12-mm round coverslips for transient transfections. Confocal z-stacks were imaged at defined zoom (63× objective, 2× zoom) and depth (0.2 μm), and they were acquired using a multiphoton confocal Zeiss LSM 510 microscope (Carl Zeiss, Jena, Germany). Cells were maintained at 37°C with a microincubator fitted to a microscope stage.

For time-lapse microscopy, live cells transfected with either pcDNA (empty vector) or pcDNA-Id1 were grown in T_{25} flasks. The cultures were transferred to a heated chamber (37°C, 5% CO_2) and imaged for 16 h with the Zeiss LSM 510 microscope, equipped with a 40× Plan-Neo DIC 1.3 numerical aperture objective. Pictures were taken every 10 min for 19 h by using the Axiocam software (Carl Zeiss).

Statistical Analysis

Student's *t* test was used where applicable. The mean percentage and SE of three independent experiments and at least 200 interphase cells or 50 mitotic cells evaluated per experiment are given. $p < 0.05$ was considered as statistically significant.

RESULTS

Stable and Transient Expression of Id1 Induce Tetraploidization and Centrosome/Centriole Amplification

We have reported previously that overexpression of Id1 is common in nasopharyngeal carcinoma (Wang *et al*., 2002). The contribution of Id1 to the pathogenesis of nasopharyngeal carcinoma is not fully defined. We first examined the effect of Id1 overexpression on chromosome instability in a telomerase-immortalized nasopharyngeal epithelial cell line (NP460hTert), which has a near-diploid karyotype. Establishment and detailed characterization of NP460hTert has been described previously (Li *et al*., 2006).

Stable expression of Id1 in NP460hTert cells resulted in 30% tetraploids, indicating an effect of Id1 on mitosis ($p < 0.0001$) (Figure 1A). Previous studies have indicated that centrosomal defects could be identified at an early premalignant stage of carcinogenesis (Pihan *et al*., 2003); centrosome amplification is also strongly associated with rapid onset of aneuploidy (Goepfert *et al*., 2002; Pihan *et al*., 2003). Furthermore, it has been reported that overexpression of Id1 could induce centrosome overduplication in human foreskin keratinocytes (Hasskarl *et al*., 2004). We first sought to examine for the presence of centrosomal aberrations in our immortalized nasopharyngeal epithelial cells stably expressing Id1 (NP460hTert-Id1), by using antibody against γ-tubulin, a major component of centrosomes, (Figure 1B). Single-nucleated cells with three or more centrosomes as well as binucleated cells with more than four centrosomes are considered abnormal. In NP460hTert-Id1 cells, abnormal numbers of centrosomes was detected in 50% of the cells ($n = 200$), but <10% in control NP460hTert cells at equivalent population doublings. To further eliminate the possibility that the observed effect of Id1 on centrosome abnormality was the result of selective outgrowth of cell clones with centrosome anomalies, transient expression of Id1 in HeLa cells was performed for examination of centrosome aberration. A flag-tagged Id1 expression vector was used for tracking the Id1-expressing cells. Ectopic expression of Id1 was confirmed by Western blotting analysis (Figure 1B). Similar to the observation in NP460hTert-Id1 cells, fourfold increase in supernumerary centrosomes was detected in HeLa cells transiently expressing Id1 (Figure 1B).

To determine whether Id1 induced extra centrosomes is due to interference on centriole homeostasis, we also performed transient transfection of Id1 into U2OS cells stably transfected with green fluorescent protein (GFP)-tagged centrin to allow direct visualization of individual centriole (Figure 1C). The effect of Id1 expression in centriole homeostasis in the U2OS cells expressing Id1 was observed at 48 h after transfection. A twofold increase of extra centrioles in single-nucleated (>4) or binucleated cells (flag-positive) cells (>8) ($n = 200$) (Figure 1C) was detected. Similar increase in centriole numbers was noted in NP460hTert+Id1 cells by immunofluorescent staining with the centrin antibody (Figure 1C).

Id1 Disrupts Bipolarity and Induces Multipolar Spindles

Ectopic Id1 expression also induced a high frequency of mitotic spindle defects in cells. Most notable was the increase in the percentage of cells with multipolar and monopolar spindles, revealed by immunofluorescent staining with α-tubulin. Twenty-six percent of the NP460hTert-Id1 cells revealed multipolar mitotic spindles and monopolar spindles ($n = 50$) (Figure 1D). Using live cell imaging, multipolar spindles were also observed in 23% of HeLa cells ($n = 50$) cotransfected with Id1 and GFP-α-tubulin (5:1 ratio), compared with 5% in the control cells ($n = 50$) (Supplemental Figure 1A). In both cell models, the frequency of mono- and multipolar spindles observed in Id1-overexpressing cells is lower than the frequency of extra centrosomes observed (Figure 1, B and C), suggesting that some centrosomes in the Id1-overexpressing cells do not possess microtubule-nucleating activity in mitosis (Supplemental Figure 1B).

Id1 Induced Binucleation May Arise as a Consequence of Defects in Cytokinesis

Quantitative analysis of the NP460hTert-Id1 cells revealed that around one third of the Id1-overexpressing cells that

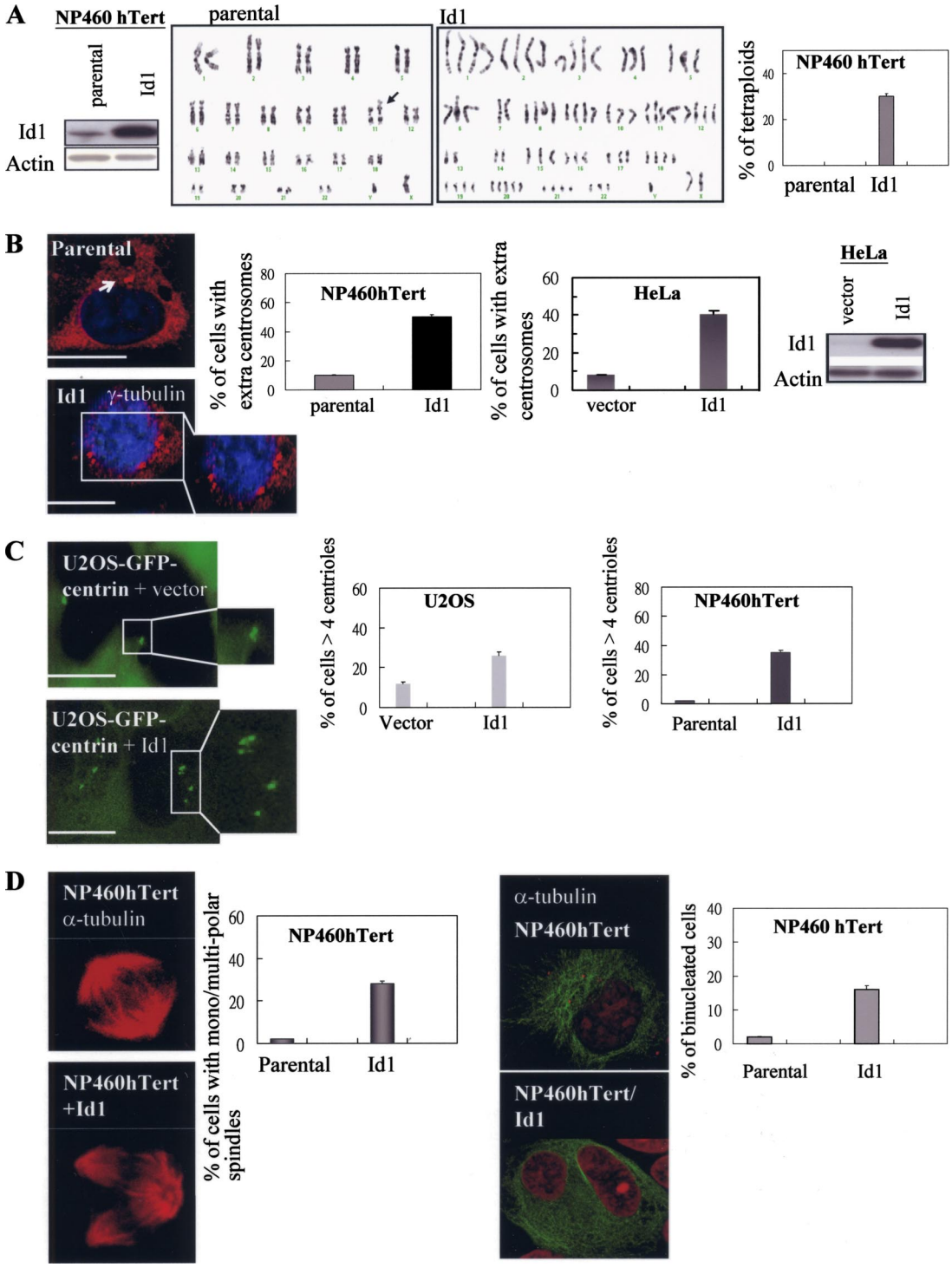

Figure 1. Stable expression of Id1 induced tetraploidization. (A) Western blot confirming high level of Id1 expression following NP460 hTert transduced with the Id1 gene. Representative karyotypes of NP460 hTert and NP460 hTert-Id1 performed by conventional cytogenetics. Histogram showing the percentage of tetraploids in NP460 hTert transduced with either the empty vector pBabe or with pBabe-Id1. (B) Stable expression of Id1 induced supernumerary centrosomes. Quantification of centrosomes in NP460hTert-pBabe and NP460hTert-Id1 cells. Immunofluorescent staining by using Alexa Fluor 568-conjugated antibody against γ-tubulin. Maximum projections of z-stacks were taken. Single-nucleated cells with more than two γ-tubulin foci and binucleated cells with more than four foci were quantified as abnormal. Transient Id1 overexpression resulted in supernumerary centrosomes. HeLa cells transiently transfected either with pcDNA3 or pcDNA3-flag-Id1 for 48 h, expression of Id1 confirmed by Western blotting. Flag-positive cells were scored for the centrosome number. Immunofluorescent staining and quantification of centrosomes were performed as described. (C) Transient transfection of flag-tagged Id1 plasmid in U2OS cells resulted in abnormal centriole number. U2OS cell line stably expressing GFP-centrin was used to allow visualization of individual centriole. U2OS cells were either transfected with pcDNA3-flag-Id1 or the empty vector. Flag-positive cells were examined for centriole number. Maximum projections of z-stacks of centrioles were taken. Histogram showing the number of extra centrioles (>4) in single-nucleated cells or (>8) in binucleated cells. Stable expression of Id1 induced supernumerary centrioles. Quantification of centrioles in

carried extra centrosomes were binucleated (Figure 1D). Conversely, binucleated cells were not common in NP460hTert-Id1 cells with normal number of centrosomes. This indicates that the extra centrosomes in the binucleated cells may result from cytokinesis defect.

Binucleated cells could be generated by cytokinesis failure or cell–cell fusion (Nigg *et al.*, 2002). To corroborate the interpretation that high level of Id1 may affect cytokinesis, time-lapse microscopy was performed in Id1-expressing HeLa cells and control cells. After cotransfecting the Id1 with the DsRed expression plasmids (at a 5:1 ratio) for 36 h, cells were followed by time-lapse microscopy for 16 h. Pictures were taken every 5 min for a 16-h duration. Mitotic cells were analyzed from metaphase cells when the condensed chromosomes lined up on the equatorial plane, to the exit of mitosis after abscission of the cleavage furrow. Id1-expressing cells exhibited a defect in mitotic exit. A prolonged transition time from anaphase to abscission of furrow was detected in Id1-expressing cells (37 5.6 min, $n = 44$), compared with (20 2.5 min; $n = 46$) in control cells ($p < 0.0001$) (Supplemental Figure 1C). These observations suggest that Id1 may interfere with the cytokinesis process leading to binucleation and tetraploidization.

Id1 Perturbs Organization of Interphase Microtubules and Integrity of Mitotic Spindles

In addition to abnormal number of spindle poles, we also detected diminished GFP intensity that represents poorly formed mitotic spindles in 12% ($n = 50$) of the Id1-transfected HeLa cells (Supplemental Figure 1D), compared with 2% in the control cells. This would imply that overexpression of Id1 also has pronounced effect on microtubule organization.

The ability of Id1 to perturb interphase microtubules has not been reported previously. Failure of microtubule polymerization was detected in 45% of the NP460hTert-Id1 interphase cells (Supplemental Figure 2A) compared with 3% in control cells ($n = 200$). The effect of Id1 overexpression on microtubule polymerization was examined by live cell imaging in HeLa cells cotransfected with Id1 and GFP-tagged α-tubulin expression plasmids (5:1 ratio). Twenty-six percent of HeLa cells transiently expressing Id1 resulted in loss of microtubule integrity (Supplemental Figure 2A).

Id1 Up-Regulates Aurora A Expression

We next sought to identify molecular event(s) responsible for the generation of the abnormal phenotypes induced by Id1. Considering the role of several mitotic kinases in the centrosome cycle, many of the Id1-induced phenotypes (tetraploidization, extra centrosomes, binucleation, monopolar/multipolar mitotic spindles) are similar to the phenotypes associated with Aurora A overexpression (Meraldi *et al.*, 2002). We further examined whether Id1 could up-regulate Aurora A expression to induce these abnormal phenotypes. Western blotting analysis of NP460hTert-Id1 cells revealed up-regulation of Aurora A expression (Figure 2A). We then examined the ability of Id1 to up-regulate Aurora A expression by transient transfection of Id1 into HeLa cells. Up-regulation of Aurora A by transient Id1 expression could also be demonstrated in HeLa cells in a dose-dependent manner (Figure 2B).

Id1 Depletion Down-Regulates Endogenous Aurora a Level and Suppresses Centrosome Amplification/ Microtubule Perturbation in Cancer Cells

To examine whether Aurora A overexpression may mediate the abnormal mitotic phenotypes observed in Id1-overexpressing cells, we performed knockdown experiments using shRNAs. An undifferentiated nasopharyngeal cancer cell line, HONE1, was chosen for this part of the knockdown study, due to its endogenous high levels of Aurora A and Id1 as well as the presence of supernumerary centrosomes (55%). Transfection of HONE1 cells with a previously characterized Id1-antisense expression vector (pcDNA3-Id1AS) (Ling *et al.*, 2003) resulted in a marked reduction in Id1 level (Supplemental Figure 2B). Id1 depletion in HONE1 cells also resulted in a decrease in the endogenous Aurora A expression level (Supplemental Figure 2B), suggesting that overexpression of Aurora A is dependent on Id1. In parallel, the percentage of HONE1 cells with extra centrosomes was reduced by fourfold and twofold, after suppression of Id1 or Aurora A, respectively (Supplemental Figure 2C), indicating that overexpression of Id1 plays an important role in mediating supernumerary centrosomes in HONE1 cells. We also reported previously that U2OS cells also have endogenous supernumerary centrioles (Gromley *et al.*, 2003) as well as high level of Id1. The number of supernumerary centrioles in U2OS cells stably expressing GFP-centrin was suppressed by 2- to 3.5-fold, when transiently transfected with shAurA alone, Id1-AS alone (Figure 2B). This set of knockdown experiments indicates the involvement of Aurora A in mediating centrosome and centriole amplification in Id1-overexpressing cells. When Id1 and shAurA plasmids were cotransfected into U2OS cells, the extra centriole phenotype was partially suppressed (Figure 2B), indicating that Id1 is mediating some other events involved in centrosome regulation.

Gene silencing studies, in HONE1 cells, were performed to examine the role of Id1 and Aurora A in association with the microtubule phenotype (Supplemental Figure 2D), which was revealed by transiently expressing GFP-tagged α-tubulin, Id1 depletion suppressed the disturbed microtubule organization in HONE1 cells by fourfold (Supplemental Figure 2D), suggesting that high level of Id1 expression is responsible for the abnormal microtubule phenotype observed. The defective microtubules of HONE1 cells could be suppressed, but to a lesser extent, when Aurora A expression was silenced (Supplemental Figure 2D). This set of data suggests that Id1-induced microtubule aberration might be mediated through other factor in addition to Aurora A.

The above-mentioned findings were confirmed in a telomerase immortalized RPEhTert epithelial cells stably expressing GFP-α-tubulin, previously used in our microtubule studies (Rosa *et al.*, 2006; Man *et al.*, 2007). Exogenous expression of Id1 in RPEhTert cells also induced abnormal interphase microtubules and monopolar mitotic spindles (Figure 2C), compared with the network-like microtubules in the control

Figure 1 (cont). NP460hTert-pBabe and NP460hTert-Id1 cells. Immunofluorescent staining using Alexa Fluor 488-conjugated antibody against centrin. Maximum projections of z-stacks were taken. Single-nucleated cells with more than four centrin foci and binucleated cells with more than eight foci were quantified as abnormal. (D) Effect of Id1 overexpression on spindle polarity in NP460hTert cells. Id1 induces multi- and monopolar spindles. Immunofluorescent staining using Alexa Fluor 568-conjugated antibody against α-tubulin. Maximum projections of z-stacks were taken. Cells with either monopolar or multipolar spindles were quantified as abnormal ($n = 50$). Id1 induces formation of binucleated cells. NP460 hTert and NP460 hTert-Id1 cells were immunofluorescent stained with α-tubulin antibody ($n = 200$). Percentage of binucleated cells was quantified. Confocal microscopy was performed as previous. Bars, 10 μm.

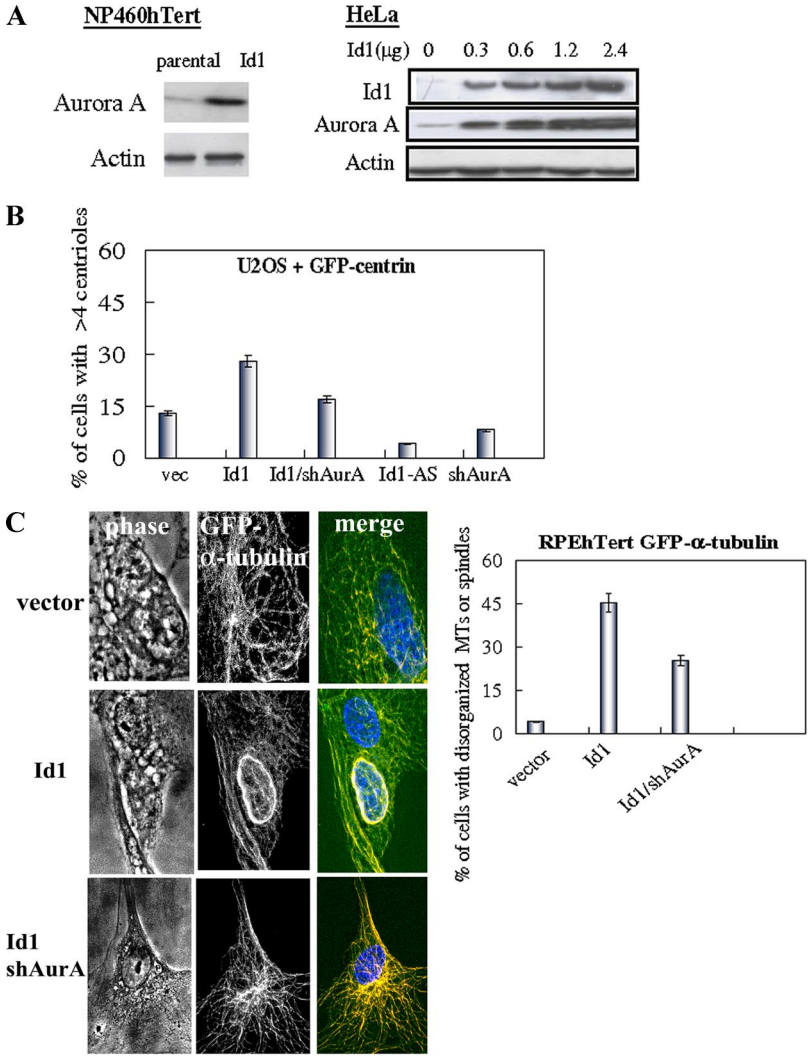

Figure 2. Aurora A mediates the mitotic defects in Id1-overexpressing cells. (A) High expression level of Aurora A in stable and transient Id1 overexpressing cells. Detection of Aurora A expression level by Western blotting in NP460hTert cells transduced with the empty vector (pBabe) or with pBabe-Id1. Expression of Aurora A in HeLa cells was detected 48 h after the cells were transfected with different doses of pcDNA3-Id1. (B) Rescue of the centriole phenotype by knocking down Aurora A/Id1 in live U2OS cells stably expressing GFP-centrin. U20S cells stably expressing GFP-centrin was cotransfected with Id1 and DsRed (5:1) to detect the proportion of cells with abnormal centriole numbers ($n > 4$); and compared with the effect of either Id1 depletion by using an antisense expression plasmid, pcDNA3-ASId1, or Aurora A knocked down using short hairpin RNA targeted specifically against Aurora kinase A, pSR-shAURKA, or expressing both pcDNA-Id1 and pSR-shAURKA. Subsequent detection, confocal microscopy, and quantification of centrioles were performed as described in Figure 1C. (C) Aurora A suppression rescued the abnormal microtubule phenotype in Id1 overexpressing RPEhTert cells. Exogenous Id1 expression in retinal pigment epithelial (RPE) cells stably expressing GFP-α-tubulin was attained by transient transfection, followed by confocal microscopy 48 h after transfection. To ensure the abnormal phenotypes were not artifacts of transfection or cytotoxic effects, cells were checked under phase-contrast microscopy for the presence of intact membrane structure. Live cells were subsequently fixed and immunostained with (DM1α) α-tubulin antibody and Alexa Fluor 568-conjugated secondary antibody to verify the origin of the distorted features. Merged images of GFP-α-tubulin and (DM1α) α-tubulin were generated using the LSM510 Meta software (Carl Zeiss). Experiments were repeated by cotransfecting pcDNA-Id1 and pSR-shAurA or transfecting with the empty vector alone. Microtubule defects in live RPE-GFP-α-tubulin cells were detected and quantified ($n = 200$) by confocal imaging as described. Bars, 10 μm.

cells. Number of cells with perturbed microtubules/spindles were suppressed by 50% when Aurora A expression was depleted by shRNA in Id1-overexpressing cells (Figure 2C). To ensure these phenotypic changes were not artifacts of DNA transfection or cytotoxic effects, cells scoring for abnormal microtubule structures were checked under phase-contrast microscopy for the presence of intact membrane structure (Figure 2C). In addition, cells were subsequently fixed and immunostained with DM1α-tubulin to verify the distorted microtubules (data not shown). Similar to the centrosome scenario, partial rescue of the microtubule phenotypes by Aurora A in both cell models overexpressing Id1 suggests that other events mediated by Id1 may be responsible for the microtubule effect.

Mechanisms Involved in the Up-Regulation of Aurora A by Id1

Because up-regulation of Aurora A seems to play a role in mediating the abnormal phenotypes, we carried a series of experiments to examine some of the plausible mechanisms involved.

Induction of Aurora A by Id1 Is Not Related to Cell Cycle

The expression of Aurora A peaks at G2/M because of the enrichment of this protein either to the centrosomes (Hirota *et al.*, 2003) or to the microtubule compartments (Lee *et al.*, 2001; Kinoshita *et al.*, 2005). Aurora A is rapidly degraded after anaphase, and it could be detected, weakly, after G1 phase of the next cell cycle (Castro *et al.*, 2002b; Taguchi *et al.*, 2002). We examined whether the induction of Aurora A expression by Id1 may be a result of cells accumulating at G2/M phase of the cell cycle. FACS analysis did not reveal significance accumulation of cells at G2/M in Id1-overexpressing HeLa cells (Figure 3A). Furthermore, elevated Aurora A expression could be detected in Id-1–expressing HeLa cells synchronized at various phases of the cell cycle, and the efficacy of successful synchronization was verified by FACS analysis (Figure 3B).

The results in Figure 3B were further confirmed by bivariate antigen flow analyses (Figure 3C). Double immunofluorescent staining, by using Aurora A antibody and PI staining of cellular DNA content, was performed in HeLa cells expressing Id1 or the vector control. Isotypic control was performed to reveal antibody background (data not shown). Each spot represents an individual cell with a specific Aurora A level (*y*-axis) and DNA content (*x*-axis). Identical reference lines were drawn in corresponding panels to assist comparison of expression levels. Consistent with the results from Figure 3B, Aurora A expression was lower at G1/S phase but higher at G2/M phase in the cells transfected with

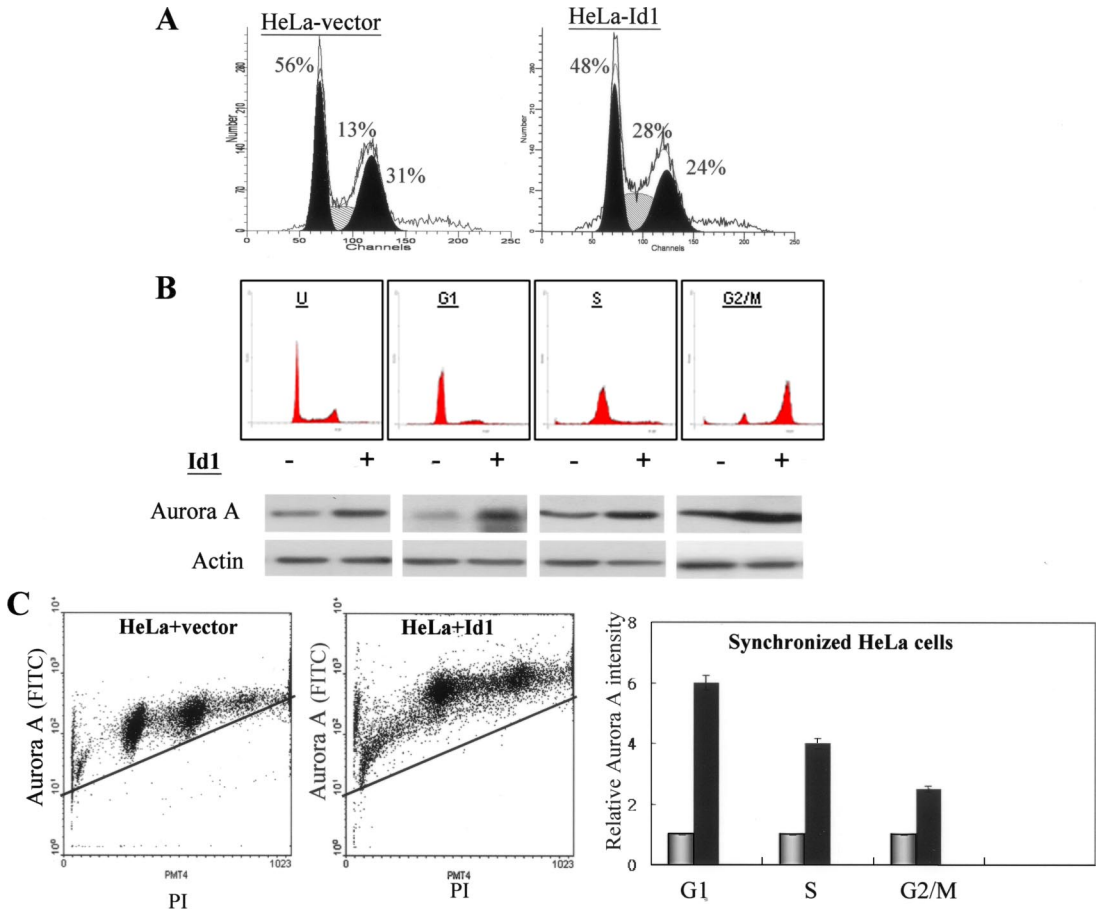

Figure 3. Id1 up-regulates Aurora A expression throughout the cell cycle. (A) FACS analysis showing the cell cycle distribution of the HeLa cells transfected with the vector control or the Id1 expression plasmid. (B) Aurora A expression levels were detected in Id1-overexpressing HeLa cells, and cells were transfected with vector control. Western blotting studies were performed in unsynchronized cells and in cells synchronized at different phases of the cell cycle by using aphidicolin and nocodazole. U denotes unsynchronized cell population. FACS analysis was used to verify successful synchronization. (C) Bivariate antigen DNA flow analysis confirmed Aurora A up-regulation by Id1 in all phases of the cell cycle. Aurora A expression and DNA content in HeLa cells expressing either Id1 or the empty vector was detected by bivariate FACS. Aurora A was detected in cycling cells by immunofluorescent staining using Aurora A and FITC-conjugated antibodies. DNA content was measured by PI staining. Each spot represents an individual cell with a specific Aurora A level (*y*-axis) and DNA content (*x*-axis).

control vector (Figure 3C). Id1-expressing cells revealed a substantial increase of Aurora A level, revealed by the FITC intensity above the identical reference lines, at all phases of the cell cycle. The mean FITC intensities of both G1 and S phase populations were at least four- to sixfold higher ($p < 0.005$) than the mean FITC intensities in the corresponding controls (Figure 3C). The mean FITC intensity of the G2/M phase population was two- to threefold higher ($p < 0.005$) than the intensity of the control (Figure 3C). Here, we show that the increased Aurora A level by Id1 is not a consequence of redistribution of cells to the G2/M phase of the cell cycle.

Gene Amplification Is Not Involved in the Elevated Aurora A Level by Id1

Our previous studies had identified amplification of 20q13.2 as a nonrandom event during immortalization in human ovarian epithelial cells, in which numerous copies of *Aurora A* were mapped to homogeneous staining regions (hsr) on chromosome 20q13 (Chung *et al*., 2005). In breast and ovarian cancer cell lines, a 2.5- to 8-fold amplification of Aurora A accounted for its high expression (Zhou *et al*., 1998). The possibility that *Aurora A* gene was amplified in NP460 hTert-Id1 cells were examined using the PAC clone (RP5843L14), which mapped to the chromosomal locus of *Aurora A*. Another PAC clone (RP5-1107C24) juxtaposed to RP5843L14 was cohybridized to metaphases as internal control. Two normal signals from each PAC clone (RP5-843L14 and RP5-1107C24) were revealed in NP460hTert (n = 200) (Figure 4A). NP460 hTert-Id1 cells did not reveal any multiple tandem gene copies mapped on 20q13.2 (Figure 4A). Two extra signals detected in NP460 hTert-Id1 cells (Figure 4A) were due to the induction of tetraploid progeny by Id1. The third extra signal was arisen from the immortalization process (Li *et al*., 2006). Our data suggest that *Aurora A* amplification is an unlikely mechanism for the elevated Aurora A level detected in NP460 hTert-Id1.

Elevated Aurora A Expression in Id1-expressing Cells Only Partially Resulted from Transcriptional Activation of the Promoter of Aurora A

We then examined whether Id1 may result in transcriptional up-regulation of Aurora A promoter activity by luciferase reporter assay. HeLa cells were cotransfected with various

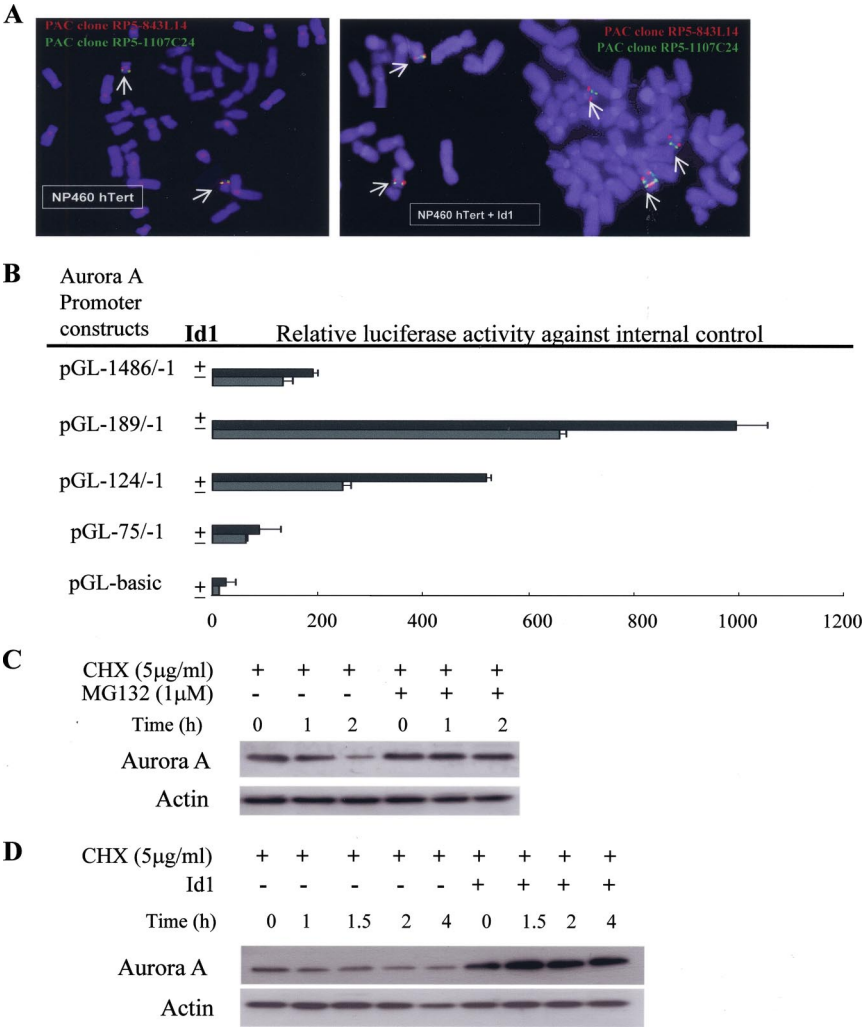

Figure 4. Mechanism(s) involved in Aurora A up-regulation by Id1. (A) Overexpression of Aurora A in NP460 hTert-Id1 cells is not a consequence of gene amplification. FISH analyses of NP460 hTert and NP460 hTert-Id1 cells by using the PAC clone (RP5-843L14), which resides at the locus of Aurora A; another PAC clone (RP5–1107C24), juxtaposed to the locus of Aurora A, was used as an internal control. The PAC clones were labeled with Spectrum red and Spectrum green, respectively, by random priming. (B) Aurora A promoter activity is mildly enhanced by Id1. Schematic representation of the 5′-truncated Aurora A promoter-luciferase constructs and their activities in HeLa cells. HeLa cells were transiently transfected with the 5′-truncated Aurora A promoter-luciferase constructs. Results were expressed as the ratio of luciferase activity in each extract relative to the extract transfected with pGL-basic. This set of result was compared with a second set of result, in which HeLa cells were cotransfected with Aurora A promoter-luciferase constructs and the Id1 expression plasmid. (C) Inhibition of proteolytic degradation of Aurora A degradation by Id1 expression. Degradation of Aurora A is mediated through the ubiquitin–proteasome pathway. Degradation profile of Aurora A protein was examined by blocking protein synthesis by cycloheximide for 2 h. The effect of proteasome inhibitor on Aurora A stability was examined, by treating the cells with cycloheximide together with 1 μM MG132. Western blotting analysis was performed on the protein pellets collected at the indicated time points. Expression of Id1 led to stabilization of Aurora A in HeLa cells. Cells were either transfected with the control vector or the Id1 expression plasmid for 48 h. The transfected cells were treated with cycloheximide after transfection for the indicated time points. The level of Aurora A was examined by Western blotting.

5′-truncated Aurora A promoter luciferase constructs (pGL1486, pGL189, pGL124, and pGL75) and the Id1 expression plasmid (or the pGL basic vector). A 1.5- to 2-fold increase in Aurora A promoter activities was observed in Id1-transfected cells, the activity being highest between −189 and −124 of the core promoter (Figure 4B). Our results suggest that Id1 may regulate the transcriptional activity of Aurora A at its core promoter between the −124 to −189 from the transcription start site. This weak increase in promoter activation (do not exceed 2-fold) in Id1-expressing cells indicates that transcriptional activation of Aurora A by Id1 may not be the mechanism involved in its up-regulation.

Id1 Affects Aurora A Degradation

Aurora A is rapidly degraded at mitotic exit by the proteasomal proteolysis pathway (Littlepage and Ruderman, 2002). We examined whether Id1 overexpression may affect this process. The endogenous turnover rate of Aurora A was detected in HeLa cells by treatment with cycloheximide, a protein biosynthesis inhibitor in eukaryotic cells. The endogenous Aurora A in HeLa cells has a rapid turnover rate with a half-life of ~2 h (Figure 4C). In line with other studies, we confirmed that degradation of Aurora A in HeLa cells could be prevented by proteasomal inhibition by using MG132 (inhibitor of the proteasomal degradation pathway) (Figure 4C). Furthermore, Aurora A could not be degraded in Id1-overexpressing cells, even in the presence of cycloheximide, indicating that Id1 overexpression resulted in stabilization of Aurora A (Figure 4D).

Aurora A and Id1 Are Not Binding Partners

Aurora A is a known substrate of the anaphase-promoting complex. This protein contains the D-box and the A-box sequences known to be required for the recognition and destruction by the activated APC/C^{Cdh1} (Pfleger *et al.*, 2001; Castro *et al.*, 2002a,b; Crane *et al.*, 2004) at mitotic exit and during the following G1 phase. To examine whether the resistance of Aurora A to proteasomal degradation by Id1 overexpression could be a result of direct interaction between Id1 and Aurora A, we performed coimmunoprecipitation studies. HeLa cells were transfected with a wild-type Id1 flag-tagged expression vector. Coimmunoprecipitation studies using the flag and Aurora A antibodies as baits failed to reveal any interaction between Id1 and Aurora A (Figure 5A).

Binding of Id1 to the Anaphase-promoting Complex Coactivator (cdh1) Affects the Stabilization of Aurora A

The APC/C^{Cdh1} targets Aurora A for the ubiquitin proteasomal-dependent degradation. The enzymatic E3 ubiquitin ligase activity of APC/C requires Cdh1 as one of the coactivators at late mitosis (Pfleger *et al.*, 2001; Littlepage and

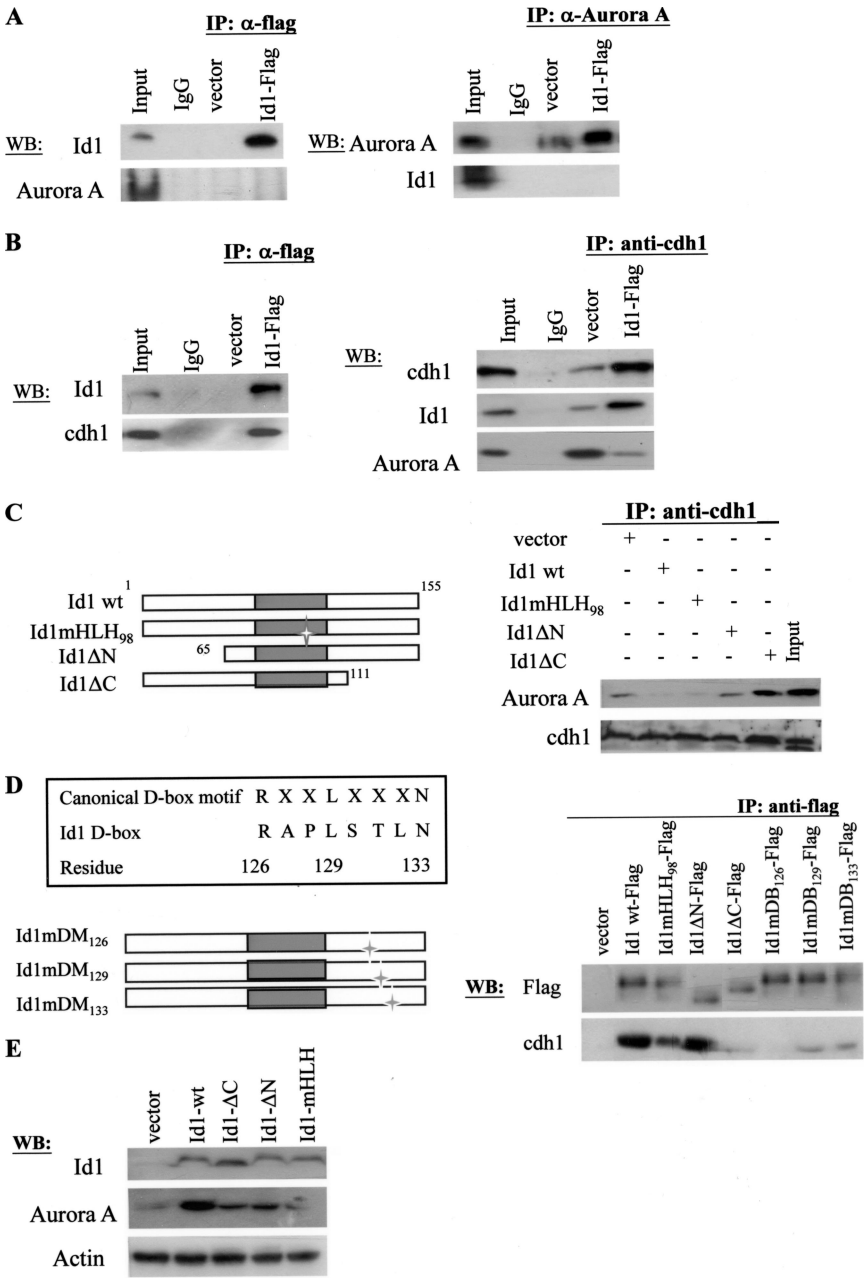

Figure 5. Id1 affects Aurora A degradation. (A) Id1 and Aurora A are not interacting proteins. HeLa cells were transfected with flag-tagged Id1 expression plasmid or the control vector. Cellular extracts were sequentially immunoprecipitated with either rabbit immunoglobulin G (IgG), anti-Flag M2, or anti-Aurora A antibodies. The immunoprecipitates were recovered on protein A/G and analyzed by Western blotting to confirm successful pull-down, and subsequent examination for the potential interactions between Id1 and Aurora A. (B) Id1 and cdh1 are novel binding proteins. Binding of Id1 to cdh1 affects Cdh1 association with Aurora A. Transfection of HeLa cells and coimmunoprecipitation studies were performed as described in A. Cellular extracts were sequentially immunoprecipitated with either rabbit IgG, anti-Flag M2, or anti-Cdh1 antibodies. The immunoprecipitates were recovered on protein A/G and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting for the presence of Id1, Cdh1, and Aurora A in the pulled-down complex. (C) Mapping of the domain of Id1 essential for Cdh1–Aurora A association. Cells were transfected with the Id1 wild-type expression plasmid and the mutants, respectively. The Id1-mHLH$_{V98G}$ comprises a point mutation in the second helix domain at amino acid 98, resulting in a substitution of V to G. The Id1-ΔN lacks the amino terminal (1-64 amino acids), whereas the Id1-ΔC lacks the carboxy terminal 44 amino acids. Transfected cell lysates were immunoprecipitated with anti-cdh1 antibody, recovered on protein A/G, and processed for SDS-PAGE and Western blotting to confirm successful pull-down as described. The pull-down complexes were subsequently examined for the presence of Aurora A by Western blotting. (D) The destruction box (D-box) motif within the C-terminal of Id1 is involved in its binding to Cdh1. Id1 D-box mutants were generated as described in text. HeLa cells were transfected with the indicated expression plasmids carrying mutations of the D-box at amino acids 126$_{R->P}$, 129$_{L->V}$ and 133 $_{N->K}$; the Id1 wild type; Id1-ΔN, Id1-ΔC, the Id1-mHLH$_{V98G}$; or the vector control expression plasmids. Transfected cell lysates were immunoprecipitated with anti-Flag M2 antibody, recovered on protein A/G, and processed for SDS-PAGE and Western blotting to confirm successful pull-down. The pulled down complexes were subsequently examined for the presence of Cdh1 by Western blotting. (E) Mapping of the domain of Id1 essential for Aurora A stabilization. HeLa cells were transfected with the Id1 wild-type expression plasmid and the mutants Id1-mHLH$_{V98G}$, Id1-ΔN, and Id1-ΔC, respectively. Western blotting was performed to monitor the effect of the Id1 domain required for stabilization of Aurora A.

Ruderman, 2002; Castro *et al*., 2002b; Crane *et al*., 2004). Cdh1 can recognize APC/C substrates by interacting with specific recognition elements (D-box) in these substrates (Kraft *et al*., 2005). It has been reported previously that Aurora A could be immunoprecipitated with Cdh1 (Crane *et al*., 2004). Stability of Aurora A could be associated with depletion of Cdh1 expression in somatic, cells whereas restoration of Cdh1 rapidly induces fall in Aurora A level (Taguchi *et al*., 2002; Crane *et al*., 2004). We then explored whether Id1 associates with Cdh1 and competes for the binding of cdh1 to Aurora A. Our coimmunoprecipitation studies revealed that Id1 and cdh1 are indeed interacting partners (Figure 5B). Excessive Id1–cdh1 interaction could inhibit the association between Aurora A and Cdh1 (Figure 5B). Hence, decreased recognition or binding of Cdh1 with Aurora A may result in stabilization of Aurora A.

Id1ΔC Fails to Induce Cdh1/Aurora A Dissociation and the D-Box Motif in the C-Terminal of Id1 Is Involved in Its Binding to Cdh1

We next investigated on the functional domain(s) of Id1 responsible for the dissociation of Cdh1 and Aurora A. Immunoprecipitation studies were performed on cells expressing the wild-type Id1 and other Id1 mutants. Decreased Cdh1 and Aurora A association was clearly observed in cells expressing the wild-type Id1 (Figure 5C). Inhibition of Cdh1

and Aurora A association was also observed in cells expressing the Id1 mutant carrying a specific mutation at residue 98 of the second helix. Deletion of the C-terminal 44 amino acids resulted in a full recovery of Cdh1/Aurora A association; indicating that the C terminus of Id1 comprises a motif that may be crucial for Cdh1/Aurora A recognition (Figure 5C). It was also noted that deletion of the N-terminal 65 amino acids resulted in weak recovery of Cdh1/Aurora A association; suggesting the possible involvement of the Id1 N terminus in Cdh1/Aurora A association.

The APC/C^{Cdh1} targets mitotic proteins for ubiquitin-mediated degradation mostly through the D-box recognition sequence/motif (RxxLxxxN) (Zachariae, 2004), and it has been reported that the Id2 protein contains this recognition sequence for interacting with the APC/C (Lasorella *et al.*, 2006). Apparently, this motif is also present in Id1 and Id4. We then focused on the C terminus (residues 126-133) of Id1 (in which the D box is located), and we examined whether this motif is essential for the interaction between Id1 and Cdh1. Three flag-tagged Id1 mutants with mutations at specific residues within the destruction box (mDB_{126}, mDB_{129}, and mDB_{133}) were generated, and their binding abilities with Cdh1 were examined (Figure 5D). We showed that these DB mutants, as well as Id1ΔC-flag, are less effective to precipitate Cdh1 than Id1wt-flag, indicating that the destruction box recognition motif (residues 126-133) is required for Id1–Cdh1 association. Results from Figure 5, B–D, suggest that Id1 promotes binding with Cdh1 at the destruction box and that it does inhibit Cdh1 to bind to its substrate, Aurora A.

If Id1 inhibits APC/C^{Cdh1}, it should thereby accumulate Aurora A at late M phase and at G1. According to the results from Figure 5D, one would expect that expression of wild-type Id1, Id1-ΔN, and Id1-mHLH, but not the Id1-ΔC (which lacks the D box) could stabilize Aurora A level. Indeed, Aurora A level was shown to accumulate in cells expressing wild-type Id1, compared with cells expressing the vector control (Figures 2A, 3, B and D, 4D, and 5E). Aurora A level was seen accumulated in cells expressing the Id1-ΔN as expected, but at a relatively lower level compared with wild-type Id1. However, our results showed that Aurora A level could still be detected in cells expressing the Id1-ΔC mutant (Figure 5E), albeit at a lower level compared with wild-type Id1; furthermore, Aurora A expression was not detected in cells expressing the Id1-mHLH mutant. These unexpected results indicate that other molecular events or motifs at the N terminus/HLH domain may possibly affect Aurora A stabilization (see *Discussion*). The D-box motif at the C terminus of Id1 that binds to Cdh1 may not be the sole mechanism in modulating Aurora A stabilization in cells.

Stabilization of Other Cdh1 Substrates Because of Excessive Id1–cdh1 Association

If Id1–Cdh1 association is involved in stabilization of Aurora A, one would also expect accumulations of other Cdh1 substrates to occur in Id1-overexpressing cells. We next examined the levels of selected Cdh1 substrates (Aurora B, Cyclin B1, Plk1, and Survivin) in Id1-overexpressing cells synchronized at either G1/S or G2/M phases of the cell cycle. Similar to the Aurora A scenario, stabilization of Aurora B, Plk1, Cyclin B1, and Survivin was detected in G1 and M phase of the Id1-overexpressing cells (Figure 6A).

Immunoprecipitation studies were then performed (Figure 5B) to examine the degree of binding between Cdh1 and the selected substrates. In agreement with our hypothesis, decreased association of Cdh1 to these substrates was generally observed in cells overexpressing wild-type-Id1 (Figure 6B), compared with the vector controls. Id1ΔC mutant, which lacks the D box, failed to block the interactions between Cdh1 and its substrates, whereas wild-type Id1,

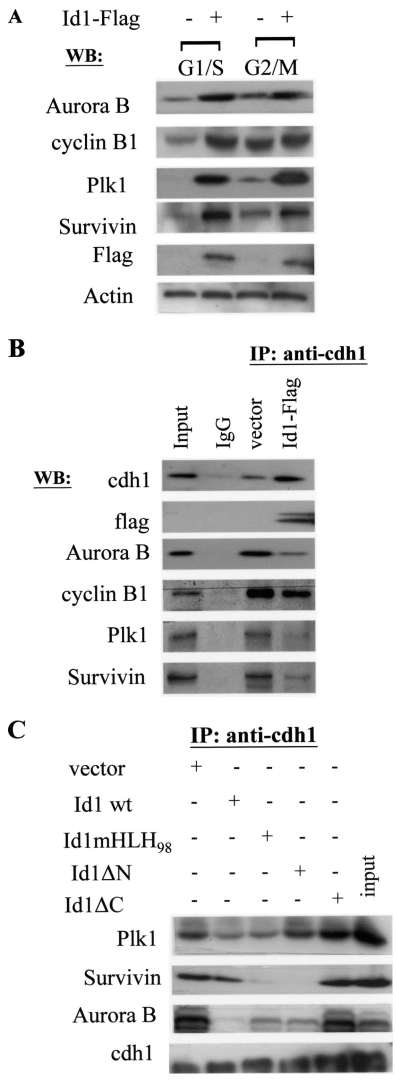

Figure 6. Stabilization of other Cdh1 substrates due to excessive Id1–cdh1 association. (A) Levels of selected cdh1 substrates in Id1-overexpressing cells were examined. Cells were transfected with either the control vector or the Id1 wild-type expression plasmid. The transfected cells (24 h after transfection) were synchronized at G1/S and G2/M phases of the cell cycle by using 6 μM aphidicolin and 0.3 μM nocodazole for the durations described. The expression levels of Aurora B, Plk1, Cyclin B1, and Survivin were analyzed in Id1-overexpressing cells and control cells by Western blotting. (B) Binding of Id1/Cdh1 masked the ability of Cdh1 to form complexes with its substrates. Transfection of HeLa cells and immunoprecipitation studies were performed as described in Figure 5, A and B. Cellular lysates were immunoprecipitated with either rabbit IgG or anti-Cdh1 antibodies. The immunoprecipitates were recovered on protein A/G and analyzed by SDS-PAGE and Western blotting for the presence of Id1, Cdh1, Aurora B, Cyclin B1, Plk1, and Survivin in the pulled-down complex. (C) Mapping of the domain of Id1 essential for stabilization of Cdh1 substrates. HeLa cells were transfected with wild-type Id1, Id1ΔC, Id1-ΔN, and Id1-mHLH mutants. The domain(s) required for Cdh1–substrate associations were examined by immunoprecipitation using the anti-Cdh1 antibody. The immunoprecipitates were recovered on protein A/G and analyzed by SDS-PAGE and Western blotting for the presence of Cdh1, Plk1, Survivin, and Aurora B.

Id1-ΔN and Id1-mHLH mutants retain their abilities to block the associations of Cdh1 to these Cdh1-specific substrates (Figure 6C). Although the general trend is clear, still it is unclear why the degree of inhibition of cdh1–substrate bindings varies in cells transfected with the mutants. That is, whereas the level of Survivin associated with Cdh1 is reduced with the Id1-ΔN mutant more effectively than the wild-type Id1, the level of Plk1 associated with Cdh1 is reduced the same as the wild-type Id1. The N terminus and the HLH domain of Id1 may also participate in regulating the recognition and binding of Cdh1 to these targets. The complexities of the molecular events involved warrant further experiments. Nonetheless, our results suggest that the destruction box motif within the Id1 C terminus is involved in inhibiting Cdh1 to interact with Aurora A and other specific substrates (Figure 6C).

DISCUSSION

Overexpression of Id1 is common in human cancers including nasopharyngeal carcinoma (Wang *et al*., 2002). This protein is commonly detected in immortalized epithelial cells that escaped senescence (Li *et al*., 2006), implying that overexpression of Id1 may be an early event during cancer development. Our data indicate that Id1 could induce multiple mitotic defects and tetraploidization.

Proper cell divisions involve tight regulation of multiple events. Perturbations in microtubule organization and dynamics, defective spindle polarity, centrosome aberrations, and altered kinetochore functions have been reported to induce abnormal mitotic phenotypes in cells. Some of which may be involved in the abnormal mitotic phenotypes induced by Id1. In line with other studies (Hasskarl *et al*., 2004, 2007), we also detected rapid induction of supernumerary centrosomes and centrioles at a significant rate in our immortalized epithelial cells transduced with Id1. Several proteins are involved in the regulation of centrosome duplication and tetraploidization. These include p53 inactivation and $p21^{Cip}$ down-regulation (Meraldi *et al*., 2002; Fujiwara *et al*., 2005). However, alteration of p53 level or mutation was not detected in NP460hTert-Id1 cells (data not shown); therefore, it is unlikely that the abnormal induction of centrosomes by Id1 is mediated through p53. In the p21 scenario, all Id proteins (Id1–4) are capable to inhibit $p21^{Cip}$ expression; however, only Id1 was reported to be able to induce centrosome amplification (Ohtani *et al*., 2001; Hasskarl *et al*., 2004). Therefore, down-regulation of $p21^{Cip}$ expression per se may not be sufficient for centrosome amplification.

The role of Aurora A in centrosome homeostasis is well documented (Marumoto *et al*., 2003; Terada *et al*., 2003; Fujiwara *et al*., 2005). Overexpression of Aurora A has been previously shown to induce centrosome amplification in vitro and in vivo (Zhou *et al*., 1998; Goepfert *et al*., 2002; Meraldi *et al*., 2002). Centrosome amplification in Aurora A-overexpressing cells could be a consequence of cytokinesis failure, resulting in the formation of binucleated cells (Meraldi *et al*., 2002; Terada *et al*., 2003). In this study, we found that many of the Id1-induced mitotic defects mirrored those of Aurora A overexpression. In this study, we have examined in details the involvement of Aurora A stabilization as a mechanism involved in mediating the abnormal mitotic phenotype induced by Id1. Depletion of Aurora A by short hairpin RNA effectively was found to rescue centrosomal aberrations and spindle defects in ≈50% of the Id1-overexpressing cancer cells. Possible mechanisms involved in the activation of Aurora A by Id1 were explored. Among which is to use FISH analyses in showing that the *Aurora A* locus was not amplified in Id1-overexpressing cells. Furthermore, we showed that Id1 could weakly activate the Aurora A promoter, but the extent of activation may only partially explain the high level of Aurora A observed in Id1-expressing cells.

The fidelity of cell division is dependent on the accumulation and orderly destruction of critical mitotic regulators. Aurora A expression is known to be controlled by degradation through the APC–ubiquitin–proteasome pathway, which occurs at the end of mitosis through the G1 phase of the following cell cycle (Walter *et al*., 2000; Taguchi *et al*., 2002). We showed that overexpression of Id1 could effectively inhibit the degradation of Aurora A in cells. The inhibition of proteolytic degradation is not the result of direct interaction of Id1 with Aurora A as revealed by the coimmunoprecipitation studies. Other mechanisms involved in stabilizing Aurora A were explored. Among them is the binding of the APC/C coactivator Cdh1 with Id1, which was shown to mask the ability of Cdh1 to form complex with Aurora A and to play an important role in stabilization of Aurora A in cells. However, an unexpected result was observed in Figure 5E, showing that deletion of the binding domain inside the C terminus of Id1 (Id1ΔC) could only result in partial destabilization of Aurora A in cells, suggesting additional events are involved in stabilization of Aurora A. A plausible explanation is that Id proteins could inactivate the ubiquitin proteasome system and affect the degradation of Aurora A in our study. Id1 could bind to various components of the 26S proteasome including the S5A subunit of the 19S proteasome (Anand *et al*., 1997; Hasskarl *et al*., 2007) and the JAB1 protein (Berse *et al*., 2004). The S5A subunit contains an ubiquitin-interacting motif domain involved in the recognition and shuttling of the polyubiquitinated substrates to the proteolytic 20S degradation chamber of the proteasome (Hershko and Ciechanover, 1998). The Id1ΔN and HLH domains are essential for binding to the S5A (Hasskarl *et al*., 2007), which interfere with the ubiquitin proteasome system. Id1 mutant that lacks the C-terminal domain (Id1ΔC) could still efficiently bind to the S5A proteasomal subunit (Hasskarl *et al*., 2007), and it may block the presentation and ubiquitination of Aurora A for proteasomal degradation, resulting in its incomplete destabilization. Hence, the results in Figure 5E reflect the combination effects of Cdh1 binding to the Id1 C-terminus D box and S5A binding to the Id1 N terminus and the HLH domain.

We further demonstrated that strong interaction of Id1 with cdh1 compromised the binding of Cdh1 to its specific substrates, such as Aurora B, Survivin, and Plk1. Stabilization of such proteins would also lead to significant mitotic perturbations such as defective microtubule–kinetochore interaction, disorganized interphase microtubules, supernumerary centrosomes, cytokinesis failure, and ploidy (Ota *et al*., 2002; Honda *et al*., 2003; Araki *et al*., 2004; Yokoyama *et al*., 2005). Some of these phenotypes were also observed in this study. This could further explain the fact that Aurora A knockdown rescued 50% of the phenotypes of Id1 overexpression and that knocking down Id1 expression could efficiently rescue these phenotypes. Preliminary observations revealed that the interphase microtubule effect induced by Id1 could be rescued by silencing Survivin and Aurora B in Id1-overexpressing cells (data not shown). Detailed mechanistic study of the disturbance of microtubule defects in mitotic exit induced by Id1 is under investigation. APC/C activity is tightly regulated by coactivators and inhibitors. In this study, Id1 can antagonize Cdh1. This competing action

of Id1 with Cdh1's bindings to its substrates is similar to another Cdh1 inhibitor Emi-1 (Reimann *et al*., 2001). Same as the Emi-1 scenario where it can competitively bind to Cdh1 and allow accumulation of substrates at G1/S, we are also uncertain whether this in vitro action of Id1 could explain the capability of Id1 to inhibit APC/C^{Cdh1} in vivo. The precise in vivo mechanism(s) warrants further investigation.

In this study, we report that Id1 could induce multiple abnormal phenotypes of mitosis and also the involvement of the Id1–Cdh1–Aurora A axis in mediating these phenotypes. Id1 overexpression is common in human cancers. The induction of abnormal mitotic phenotypes could possibly lead to aneuploidy commonly observed in malignant tumors. Our results support the role of Id1 in the induction of mitotic dysregulation in early-staged carcinogenesis and in facilitating tumor progression.

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