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Phosphorylation of NuMA by Aurora-A Kinase in PC-3 Prostate Cancer Cells Affects Proliferation, Survival, and Interphase NuMA Localization

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ABSTRACT

Aurora-A is a serine/threonine kinase that has oncogenic properties in vivo. The expression and kinase activity of Aurora-A are up-regulated in multiple malignancies. Aurora-A is a key regulator of mitosis that localizes to the centrosome from the G2 phase through mitotic exit and regulates mitotic spindle formation as well as centrosome separation. Overexpression of Aurora-A in multiple malignancies has been linked to higher tumor grade and poor prognosis through mechanisms that remain to be defined. Using an unbiased proteomics approach, we identified the protein nuclear mitotic apparatus (NuMA) as a robust substrate of Aurora-A kinase. Using a small molecule Aurora-A inhibitor in conjunction with a reverse in-gel kinase assay (RIKA), we demonstrate that NuMA becomes hypo-phosphorylated in vivo upon Aurora-A inhibition. Using an alanine substitution strategy, we identified multiple Aurora-A phospho-acceptor sites in the C-terminal tail of NuMA. Functional analyses demonstrate that mutation of three of these phospho-acceptor sites significantly diminished cell proliferation. In addition, alanine mutation at these sites significantly increased the rate of apoptosis. Using confocal immunofluorescence microscopy, we show that the NuMA T1804A mutant mis-localizes to the cytoplasm in interphase nuclei in a punctate pattern. The identification of Aurora-A phosphorylation sites in NuMA that are important for cell cycle progression and apoptosis provides new insights into Aurora-A function. J. Cell. Biochem. 114: 823–830, 2013.

KEY WORDS: Aurora-A; NuMA; APOPTOSIS; CELL PROLIFERATION; PROSTATE CANCER; CANCER THERAPY

The Aurora family of serine/threonine protein kinases regulates centrosome formation and the nuclear cycle in all metazoans as well as yeast [Glover et al., 1995; Marumoto et al., 2002]. Aurora kinases have been shown to play important roles in mitotic processes including chromosome condensation, spindle dynamics, kinetochore–microtubule interaction, chromosome orientation, and establishment of the metaphase plate [Glover et al., 1995; Marumoto et al., 2002]. Aurora-A controls mitotic centrosome and spindle assembly, and its expression and localization is consistent with its function as a mitotic kinase in centrosome regulation and separation [Kimura et al., 1997]. Aurora kinases are evolutionarily conserved proteins. All mammals have three genes, termed *Aurora-A*, *-B*, and *-C*. *D. melanogaster*, *C. elegans*, and *X. laevis* have paralogs of Aurora-A and -B, whereas *S. cerevisiae* has only one *Aurora* gene that is most closely related to mammalian *Aurora-A*. Aurora kinases have different subcellular localization patterns and play distinct roles during the cell cycle [Kimura et al., 1998; Hannak et al., 2001; Berdnik and Knoblich, 2002].

Aurora-A expression and localization is consistent with its function as a mitotic kinase. Aurora-A localizes to the centrosomal region during late G2 and mitosis where it is implicated in centrosome separation and maturation, and also regulates mitotic spindle assembly. Perturbing Aurora-A activity in flies leads to defective centrosome separation and maturation, resulting in formation of monopolar mitotic spindles and defects in cell cycle progression [Glover et al., 1995]. Ectopic Aurora-A expression leads

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to centrosome amplification, chromosome instability, and aneuploidy [Zhou et al., 1998]. Moreover, Aurora-A is an essential kinase in early embryonic development, and Aurora-A deficiency in mouse embryonic fibroblasts causes delayed mitotic entry and accumulation of monopolar spindle cells in early prophase [Cowley et al., 2009].

Aurora-A overexpression in primary breast and colon tumor samples provided the first evidence of a potential contribution in cancer [Sen et al., 1997; Bischoff et al., 1998]. Subsequent studies confirmed chromosome 20q13 amplification or Aurora-A overexpression in other tumor types, including pancreatic, ovarian, gastric, and prostate cancers [Buschhorn et al., 2005; Marumoto et al., 2005]. Aurora-A overexpression has been reported as an early pathological event in cancer progression in patients and in animal models [Buschhorn et al., 2005]. Other studies revealed a correlation between Aurora-A overexpression and higher tumor grade as well as poor prognosis [Buschhorn et al., 2005; Bufo et al., 2010]. Despite its well-characterized activity, to date, few Aurora-A substrates have been identified [Barr and Gergely, 2007]. Recently, new compounds that inhibit Aurora-A kinase activity have been identified [Yan et al., 2011]. For example, the small-molecule inhibitor VX-680 has shown promising results in vitro and in vivo [Tyler et al., 2007]. This inhibitor blocks cell proliferation, induces accumulation of cells with higher than 2N DNA content, and induces cell death in vitro. VX-680 treatment also leads to slower growth rates and regression of colon cancer xenografts in vivo [Harrington et al., 2004]. The importance of Aurora-A in cell cycle progression, the correlation with tumor progression, and enhancement of apoptosis in inhibitor-treated cancer cell lines make this kinase a promising target in cancer therapy [Jeet et al., 2012]. Currently, multiple Aurora-A inhibitors are in clinical trials and have shown promising therapeutic outcomes. For example, PHA-739358 (Danusertib) has shown disease stabilization in 24% of participants in a phase I study [Steeghs et al., 2009].

The importance of Aurora-A in several cellular processes has been well established, however, Aurora-A targets and their sites of phosphorylation remain to be defined. In an attempt to discover Aurora-A substrates, we combined the reverse in-gel kinase assay (RIKA) and small-molecule Aurora-A inhibitor treatment of cancer cells [Li et al., 2007]. Using this approach, we identified nuclear mitotic apparatus (NuMA) as an Aurora-A inhibitor-responsive substrate in the PC-3 prostate cell line. Using an in vitro kinase assay approach, we identified multiple Aurora-A phospho-acceptor sites in the C-terminal globular domain of NuMA. Furthermore, using cell-based assays, we identified specific Aurora-A phospho-acceptor sites in NuMA that are important for cell cycle progression and apoptosis.

MATERIALS AND METHODS

REAGENT AND PLASMIDS

Aurora-A and a clone encoding 1,196 amino acids of the C terminus of NuMA was purchased from American Type Culture Collection and Genecopoeia, respectively. 6X His-tagged Aurora-A and NuMA-CT were created by PCR and subcloned into expression plasmids pQE-80L (Qiagen). YFP and HA-tagged NuMA have been described. Alanine substitution mutations of NuMA were prepared with the QuikChange site-directed mutagenesis protocol and were confirmed by sequence analysis.

CELLS, CELL CULTURE, AND TRANSFECTION

The PC-3 cell line was purchased from American Type Culture Collection. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. Transfections were performed using LipoD293^{TM} Reagent (SignaGen Labratories) or TurboFect^{TM} in vitro Transfection Reagent (Fermantas). Cell lines were tested and shown to be mycoplasma-free. In addition, cells were maintained in the presence of Plasmocin^{TM} Prophylactic (5 μg/ml; InvivoGen).

REVERSE IN-GEL KINASE ASSAY AND IN VITRO KINASE ASSAY

To prepare an ~30 ml RIKA SDS–PAGE gel containing 25–50 μg/ml Aurora A, 750–1,500 μg of recombinant Aurora A dissolved in 10.5 ml 8 M urea, 250 mM imidazole, 100 mM Na_2HPO_4 (pH 7.5), was mixed with 7.5 ml 1.5 M Tris, 6 M urea (pH 8.8), 12 ml 30% acrylamide/bis (37.5:1; Bio-Rad) solution, 150 μl 20% SDS, 150 μl 1% APS, and 30 μl TEMED. After electrophoresis, RIKA gels were washed twice for 30 min each with 200 ml 20% isopropanol, 50 mM Tris (pH 7.5) to remove SDS, then with 50 mM Tris (pH 7.5), 2 mM β-mercaptoethanol twice for 30 min each at room temperature (18–24°C). Gels were then incubated in 6 M Urea, 20 mM Tris (pH 7.5) 20 mM $MgCl_2$ twice for 15 min each. Gels were then carried through a descending urea gradient (6, 3, 1.5, and 0.75 M) at 4°C by replacing half the buffer in the container with refolding buffer (50 mM Tris (pH 7.5), 2 mM β-mercaptoethanol, 0.05% tween 20) at 15 min intervals. After incubating in refolding buffer three times for 15 min each, followed by overnight incubation in refolding buffer at 4°C, gels were incubated in 200 ml 20 mM Tris (pH 7.5), 20 mM $MgCl_2$ for 30 min at room temperature. In-gel kinase reactions were performed at room temperature in 20 mM Tris (pH 7.5), 20 mM $MgCl_2$, 2 mM DDT containing 500 mCi γ-^{32}P-ATP (9,000 Ci/mmol; PerkinElmer). To stop the reaction and remove the unlabeled γ-^{32}P-ATP, gels were washed twice with 5% TCA, 1% Na_2HPO_4, and H_2O until no isotope was detected in the buffer using a hand-held Geiger counter. Gels then were dried using cellophane membrane and autoradiograms were obtained from dried gels [Li et al., 2007]. An in vitro Aurora-A kinase assay was performed as described [Li et al., 2006].

WESTERN BLOT

Western blot analyses followed standard protocols. Anti-HA rat monoclonal antibody was from Roche Applied Sciences (1:1,000), anti-actin was from Sigma (1:10,000), Rabbit polyclonal anti-NuMA antibody (1:250) was a gift from Dr. Don Cleveland.

IMMUNOFLUORESCENCE

PC-3 cells were grown on glass coverslips and transiently transfected as described above. Cells were washed three times with phosphate-buffered saline (PBS), fixed with 2% paraformaldehyde (PFA) for 15 min, and permeabilized with 0.2% Triton X-100 in PBS for 5 min. Then, the coverslips were washed again with PBS and cells were stained with DAPI and mounted for examination. Fluorescence imaging was performed using a Leica TCS 4D Scanning Confocal Light Microscope.

CELL CYCLE ANALYSIS

Cell cycle status of transiently transfected cultures was determined by flow cytometry. Cells were fixed with 70% ethanol at −20°C overnight, treated with 10 μg/ml RNase, and stained with 100 μg/ml propidium iodide. Cellular DNA content was examined using CyAn ADP flow cytometer (Beckman Coulter) and data were analyzed using Summit v4.3 software.

CELL PROLIFERATION ASSAYS

PC-3 cells were transiently transfected as described above in a 96-well multiplate and 1 μCi [^{3}H]thymidine was added to each well after transfection. After 24, 48, and 24 h samples were harvested with cell harvester. Filter mats were sealed in plastic bags with 4 ml of Betaplate scintillation fluid (PerkinElmer) and [^{3}H]thymidine incorporation was measured using a liquid scintillation counter (PerkinElmer).

RESULTS

IDENTIFICATION OF NuMA AS AN Aurora-A SUBSTRATE

To identify potential Aurora-A substrates in the human proteome, we developed a RIKA using recombinant human Aurora-A produced in bacteria. The RIKA is a robust assay that facilitates the discovery of physiological kinase substrates [Li et al., 2007]. In this assay, a protein kinase is polymerized throughout a denaturing polyacrylamide gel, which is then used to resolve a tissue or cell protein extract. Restoration of kinase activity and substrate structure followed by an in-gel kinase reaction in the presence of [^{32}P]-ATP allows phosphorylation of potential kinase substrates throughout the gel. Autoradiography reveals the positions of potential kinase substrates that can be extracted from the gel and identified by liquid chromatography tandem mass spectrometry (LC/MS^2).

RIKA identifies the non-phosphorylated substrate pool of each kinase substrate in a complex extract. Pre-treatment of cells with a specific small molecule inhibitor increases the extent of the non-phosphorylated pool of true physiological kinase substrates, and facilitates their discovery in a RIKA [Li et al., 2007]. To identify physiological Aurora-A substrates, we treated PC-3 cells with the Aurora-A inhibitor VX-680, which, at 0.6 nM, is selective for Aurora- A [Harrington et al., 2004]. PC-3 cells were exposed to VX-680 for 0, 9, and 30 h, and whole-cell protein extracts were prepared and analyzed in two-dimensional (2D) Aurora-A RIKAs. These analyses revealed hypo-phosphorylation of potential Aurora-A substrates in response to Aurora-A inhibition in PC-3 cells (Fig. 1). Phosphorylation signal derived from multiple species with varying molecular weight and pI was increased after VX-680 treatment (Fig. 1). Since RIKA detects the portion of the substrate pool that is not phosphorylated in vivo, these data demonstrate that an Aurora-A RIKA is capable of detecting inhibitor-responsive substrates. Moreover, the extent of substrate hypo-phosphorylation increased with increasing inhibitor exposure time (Fig. 1). These data suggest that potential Aurora-A substrates detectable in a RIKA accumulated in a hypo-phosphorylated state in vivo in the presence of VX-680.

Multiple Aurora-A-inhibitor responsive species were observed in Aurora-A 2D RIKA gels after PC-3 VX-680 treatment. To identify

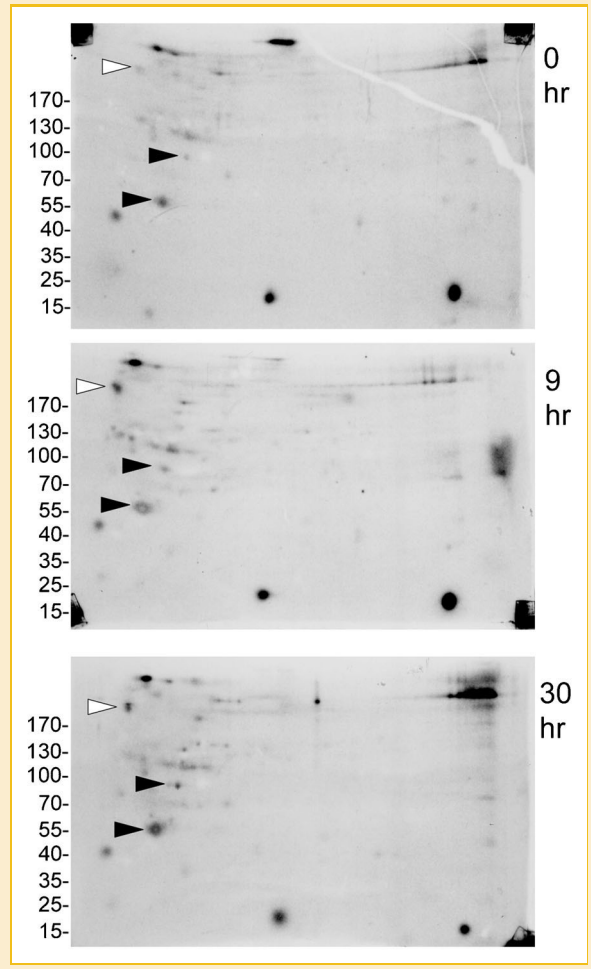

Fig. 1. Aurora-A substrate phosphorylation signal increases an Aurora-A RIKA gel after Aurora-A inhibitor treatment. PC-3 cells at 60–70% confluence were treated with 0.6 nM VX-680 for 9 and 30 h. Three hundred micrograms of cell lysate were analyzed by 2D Aurora-A RIKA. Aurora-A substrate phosphorylation signal increased with increasing time of drug treatment. Proteins that showed higher phosphorylation signals (arrowheads) were excised from the gel and analyzed by LC–MS/MS. In this experiment, NuMA (white arrowheads) was identified as an Aurora-A substrate.

inhibitor-responsive Aurora-A substrates from this kinetic analysis, we selected a high molecular weight, slightly acidic protein of ~200 kDa that underwent rapid, extensive and sustained dephosphorylation upon VX-680 exposure and also demonstrated a response that was consistent with the time of Aurora-A inhibition (Fig. 1). This high-molecular weight protein (Fig. 1) was excised from the RIKA gel, trypsinized, and analyzed by liquid chromatography tandem mass spectrometry (LC/MS^2). As expected, multiple protein identities were identified during (LC/MS^2) analysis. Beginning with the top ten identified proteins with the highest Xcorr and probability score, candidates that did not match the observed pI and molecular weight (MW) were first culled. To rank the remaining five potential substrates, we considered the known pattern of Aurora-A subcellular localization. Since Aurora-A is present in the centrosomal region, we considered it likely that its physiological substrates would also be present in the same subcellular compartment. To this

end, the remaining five potential substrate identities were searched against the centrosomal protein database, which encompasses a curated list of human proteins known to localize within the centrosome either as structural components or as centrosome visitors [Nogales-Cadenas et al., 2009]. Based on these considerations, we determined NuMA to have the highest probability of being a physiological Aurora-A substrate among the remaining candidate proteins identified in the high-molecular weight species (Fig. 1).

NuMA is a 236 kDa nuclear protein, which plays important roles during mitosis. NuMA has been demonstrated to include globular head and tail domains separated by a discontinuous alpha-helical coiled-coil domain. Interrogation of the NuMA amino acid sequence with the Gps2.1 kinase substrate prediction algorithm [Xue et al., 2008] revealed the presence of eleven potential phospho-acceptor sites for Aurora-A kinase in the globular tail domain of NuMA, and five others in the coiled-coil domain (Supplementary Table I). Since the in silico analysis predicted the existence of multiple Aurora-A potential phospho-acceptor sites in the globular tail domain of NuMA, we cloned a 63 kDa C-terminal fragment of NuMA including the distal segment of the alpha-helical region and the globular tail of NuMA into a bacterial expression vector for further analyses. To determine whether NuMA could serve as a substrate for Aurora-A, an in vitro kinase assay and an Aurora-A RIKA were performed using the recombinant 63 kDa C-terminal fragment of NuMA and Aurora-A in the presence of γ-^{32}P-labeled ATP. Both assays revealed robust in vitro phosphorylation of the 63 kDa C-terminal NuMA fragment (Fig. 2A,B). Phosphorylated C-terminal recombinant NuMA derived from an in vitro kinase assay was resolved on a polyacrylamide gel, excised, in-gel digested by trypsin, and analyzed by (LC/MS^2). Mass spectrometric analyses demonstrated that Aurora-A phosphorylates C-terminal NuMA on T1804, T1811, T1812, S1887, S1969, S2047, T 2084, and S2087. As these analyses of Aurora-A phosphorylation of NuMA were in progress, NuMA was reported to be a potential Aurora-A substrate [Kettenbach et al., 2011].

Based on the in silico identification of potential Aurora-A phosphorylation sites and in vitro mass spectrometry data, an alanine substitution library including T1804A, (T1811A/T1812A), (S1883A/S1884A), S1991A, S1887A, S2047A (T2084A/S2087A), and (T1804A/S2047A) mutants was generated (Supplementary Table I) to determine the effect of eliminating these NuMA phospho-acceptor sites on Aurora-A phosphorylation of NuMA. The recombinant C-terminal NuMA alanine substitution mutants were then analyzed in Aurora-A RIKAs and in vitro kinase assays (Fig. 2A,B and Supplementary Fig. 1). In vitro kinase assays revealed that mutations T1804A and S2047A diminished phosphorylation of NuMA by Aurora-A kinase. Also, mutations at S1883A/S1884A, T1811A/T1812A, S1991A, S1887A and T2084A/S2087A reduced phosphorylation of NuMA by Aurora-A kinase. Aurora-A RIKAs showed that alanine substitution mutations of NuMA at T1804A, S2047A, and the double mutant T1804A/S2047A abolished the Aurora-A phosphorylation of NuMA. Aurora-A RIKA phosphorylation signal decreased when NuMA was mutated at S1883A/S1884A, S1991A, S1887A, and T2084A/S2087A (Fig. 2 and Supplementary Fig. 1). These in vitro studies demonstrate existence of multiple Aurora-A phospho-acceptor sites in the C-terminal globular tail of NuMA.

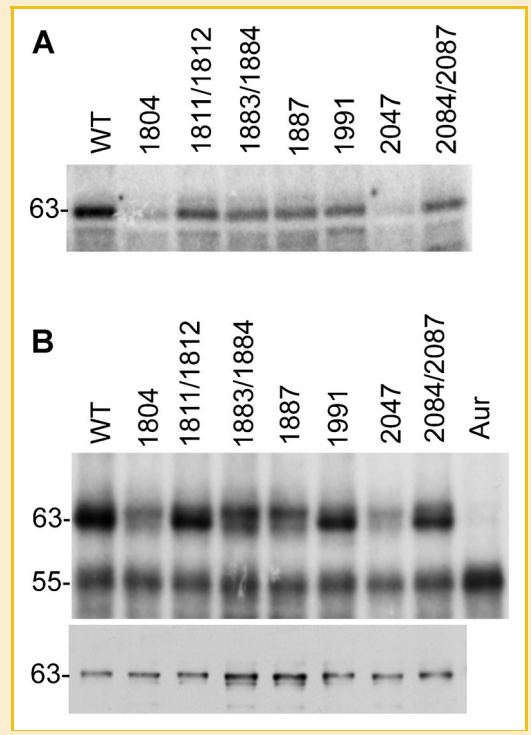

Fig. 2. Alanine substitution mutations of NuMA showed lower phosphorylation signal in Aurora-A RIKAs and in vitro kinase assays. A: WT recombinant NuMA and NuMA alanine substitute mutations were analyzed in Aurora-A RIKAs. Mutant T1804A and S2047A showed the lowest phosphorylation signal compared to WT NuMA. B: In vitro kinase assay using recombinant Aurora-A and NuMA showed phosphorylation signal reduction in NuMA alanine substitution mutants compared to WT NuMA. Recombinant WT NuMA and NuMA alanine substitute mutants were phosphorylated in vitro using Aurora-A and ^{32}P-γ-ATP. Mutant T1804A and S2047A showed the lowest phosphorylation signal compare to WT. C: Lower panel shows a Western blot analysis using anti-NuMA antibody performed to quantify proteins used in the RIKA and in vitro kinase assay.

PHOSPHORYLATION OF NuMA BY Aurora-A IS IMPORTANT FOR CELL SURVIVAL

Animal studies of carcinogen-induced malignancies revealed overexpression of Aurora-A at an early stage of tumor development [Goepfert et al., 2002]. In addition, analyses of clinical samples showed Aurora-A overexpression as an early pathological event in human prostate and ovarian tumorigenesis [Gritsko et al., 2003; Buschhorn et al., 2005]. Moreover, several studies indicated an association between Aurora-A overexpression and higher tumor grade and poor prognosis [Gritsko et al., 2003]. Aurora-A overexpression also disrupts the DNA-damage-induced G2 checkpoint, and in paclitaxel-treated cells, it disturbs the spindle checkpoint and causes cells to enter anaphase with defective spindles. This ultimately leads to paclitaxel-induced apoptosis resistance in those cells [Anand et al., 2003]. Aurora-A inhibitor treatment also increases apoptosis in multiple tumor cell lines [Huck et al., 2010]. In animal models, apoptosis associated with Aurora-A

overexpression of Aurora-A was blocked by wild type p53 [Vogel et al., 2004].

To determine the relationship between Aurora-A phosphorylation of NuMA and the cell death machinery, we tested the ability of NuMA alanine substitution mutants to induce apoptosis. Proliferating PC-3 cells transiently transfected with vectors expressing full-length HA-tagged WT or mutant NuMA isoforms were harvested and propidium iodide stained 72 h post-transfection. Overall increases in apoptosis were observed in response to the mutation of Aurora-A phospho-acceptor sites in NuMA. Cells transfected with NuMA mutants T1804A, T1811A/T1812A, S1883A/S1884A, S1991A, and T2084A/S2087A revealed a twofold increase in apoptosis compared with cells transfected with wild-type NuMA (Fig. 3). No effects on cell cycle distribution were observed upon transfection of any of the mutants. Western blot analysis showed similar expression levels of wild-type and mutant NuMA isoforms (Supplementary Fig. 2). Notably, no increase in apoptosis was observed when cells were transfected with the NuMA double mutant T1804A/S2047A. Based on in vitro kinase studies, these two sites are the major Aurora-A phospho-acceptors in the globular tail of NuMA.

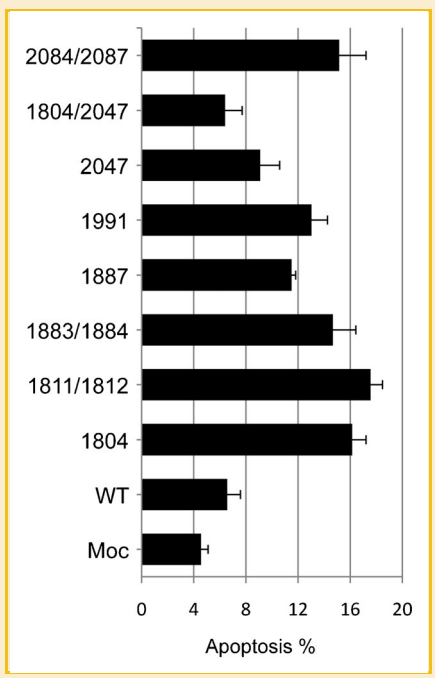

Fig. 3. PC-3 cells transfected with NuMA alanine substitution mutants are more susceptible to apoptosis. PC-3 cells were seeded at a density of 2×10^5 cells/well in 6-well plates. After 24 h incubation, cells were transfected with 4 μg of pK3H-NuMA, WT and mutants T1804A, T1811A/T1812A, S1883A/S1884A, S1887A, S1991A, S2047A, T2084A/S2087A, and T1804A/S2047A. Apoptosis was quantified by propidium iodide assay 72 h post-transfection. Apoptosis in all mutants except S2047A and T1804A/S2047A was significantly higher than WT ($P < 0.05$). Mutants T1804A, T1811A/T1812A, S1883A/S1884A, S1991A, and T1804A/S2047A showed a twofold increase in apoptosis compare to WT and mock transfected cells. Apoptosis did not increase when cells were transfected with the double mutant T1804A/S2047A. No other significant changes in cell cycle distribution were detected. Western blot analysis using anti-HA antibody showed similar expression levels of wild-type and mutant NuMA (Supplementary Fig. 2).

MUTATION OF Aurora-A PHOSPHO-ACCEPTOR SITES LEAD TO CELL PROLIFERATION DELAY

Aurora-A is an essential protein for centrosome maturation, cell cycle progression and mitotic entry [Marumoto et al., 2002]. Aurora-A siRNA or antibody depletion in HeLa cells lead to a delay in mitotic entry or mitotic progression [Marumoto et al., 2002; Hirota et al., 2003]. Also, Aurora-A depletion in *C. elegans* embryos leads to a mitotic-entry delay [Schumacher et al., 1998]. Additionally, Aurora-A inhibitors disrupt the cell cycle and inhibit cell proliferation in a broad range of cancer cells [Harrington et al., 2004].

Given the importance of Aurora-A during mitotic progression, we investigated the effect of Aurora-A phosphorylation of NuMA on cell proliferation using a [^{3}H]thymidine uptake assay. Radiolabeled thymidine was added to PC-3 cells after transient transfection with wild-type and mutant full-length NuMA. PC-3 cells transfected with NuMA mutants T1804A, T1811A/T1812A, S1883A/S1884A revealed a 65–75% reduction in cell proliferation and NuMA mutants S1887A, S1991A, S2047A, and T2084A/S2087A showed a 35–45% reduction compared to wild-type and mock-transfected cells 24 h post-transfection (Fig. 4). The cell proliferation reduction indicates the importance of Aurora-A phosphorylation of NuMA at

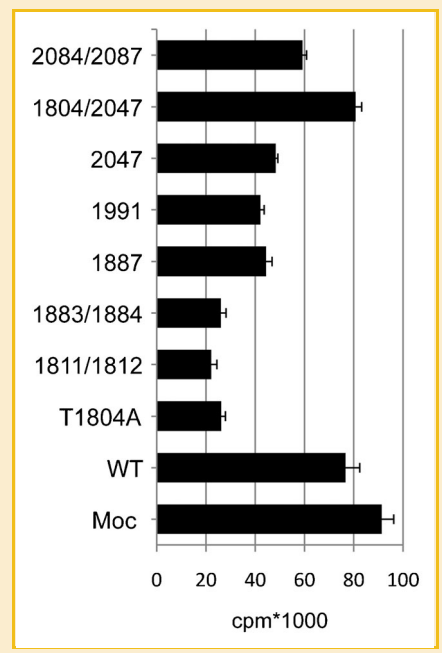

Fig. 4. Phosphorylation of NuMA by Aurora-A is important for cell proliferation. PC-3 cells were seeded at a density of 1×10^4 cells/well in the 96-well plates. After 24 h incubation, cells were transfected with 0.2 μg of pK3H-NuMA. Proliferation of PC-3 cells transiently transfected with WT and mutant NuMA T1804A, T1811A/T1812A, S1883A/S1884A, S1887A, S1991A, S2047A, T2084A/S2087A, and T1804A/S2047A for 24 h were assessed by [^{3}H]thymidine incorporation. Data shown are representative of four independent experiments. Western blot analysis using anti-NuMA antibody showed similar expression levels have wild-type and mutants NuMA (data not shown). Rates of cell proliferation in all mutants except T2084A/S2087A and T1804A/S2047A are statistically significantly different from WT ($P < 0.05$).

T1804A, T1811A/T1812A, and S1883A/S1884A sites during PC-3 cell proliferation. These residues (1,804–1,884) are located in the C-terminal tail of NuMA and are positioned adjacent to the microtubule (MTs) (1,900–1,971) and LGN (1,878–1,910) binding sites of NuMA [Du et al., 2002]. However, no cell proliferation rate reduction was observed when cells were transfected with the double mutant T1804A/S2047A. Also, apoptosis in transfected cells with this double mutant was not greater than apoptosis of WT transfected cells.

PHOSPHORYLATION BY Aurora-A AFFECTS NuMA NUCLEAR LOCALIZATION

To define the subcellular localization of NuMA mutants, PC-3 cells transiently transfected with full-length YFP-tagged wild-type NuMA and mutants were analyzed by immunofluorescence microscopy (IF). Distinct subcellular localization was observed in cells transfected with the NuMA mutants T1804A or TS1804A/2047A compared to those transfected with wild-type NuMA. IF analyses showed that wild-type NuMA was localized almost exclusively to interphase nuclei in transfected cells, however, we occasionally observed cytoplasmic NuMA localization. In contrast, the T1804A or TS1804A/2047A-transfected cells revealed a 55–60% increase in cytosolic NuMA localization (Fig. 5 and Supplementary Fig. 3). In addition, the T1804A or TS1804A/2047A NuMA mutant was not uniformly distributed in the cytosol, and formed filament-like clusters (Fig. 5). These observations suggest that phosphorylation of NuMA at threonine 1804 by Aurora-A plays a critical role in efficiently targeting NuMA in interphase nuclei. These observations, together with previous studies, reveal that NuMA phosphorylation significantly affect its subcellular compartmentalization [Compton and Luo, 1995; Kettenbach et al., 2011].

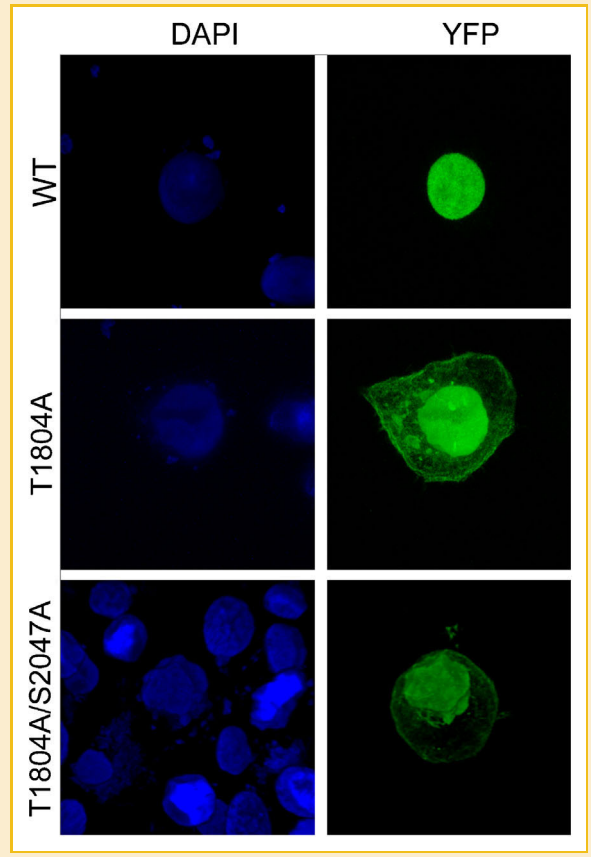

Fig. 5. Immunofluorescence microscopy shows mis-localization of mutant NuMA T1804A. PC-3 cells were seeded at a density of 2×10^5 cells/well (on glass coverslips) in the 6-well plates and after 24 h incubation were transfected with 4 μg YFP-NuMA WT or mutants. NuMA mutants T1804A and double mutant T1804A/S2047A showed 55–60% increases in cytosolic NuMA localization when compared with WT. The number of cells examined is indicated in Supplementary Figure 3. These mutants formed filament-like clusters outside the nuclei. No significant difference between LNCap and PC-3 cell lines in respond to mutation of Aurora-A phospho-acceptor sites in C-terminal tail of NuMA was observed (Supplementary Figs. 4 and 5).

DISCUSSION

Aurora-A overexpression has been reported as an early pathological event in cancer progression both in patients and animal models. We report that Aurora-A phosphorylates the C-terminal tail of NuMA at multiple sites and identify specific sites important for cell proliferation, cell survival, and NuMA nuclear localization.

Cell proliferation rates diminished when PC-3 cells were transfected with NuMA mutants T1804A, T1811A/T1812A, and S1883A/S1884A. These residues (1,804–1,884) are located adjacent to the microtubule binding domain (1,900–1,971) and the LGN (1,878–1,910) binding site of NuMA. NuMA interacts with microtubules and LGN during mitosis and abolishing these interactions leads to abnormal mitosis [Du et al., 2002]. For example, multiple studies have shown that interaction between NuMA and microtubules is essential for the astral microtubule formation and spindle pole organization during mitosis [Gaglio et al., 1995; Merdes et al., 1996]. Additionally, pre-mitotic NuMA depletion in vertebrate cells causes mitotic arrest, and likewise, depletion of NuMA during metaphase induces spindle collapse [Compton and Cleveland, 1994]. The proximity of Aurora-A phospho-acceptor sites to the microtubule interaction domain raises the possibility that NuMA–microtubule interaction may be regulated by Aurora-A activity. The LGN protein is another NuMA partner that can affect cell proliferation. It is known that in LGN-dependent cell division, disruption of NuMA–LGN interaction results in spindle mis-orientation and abnormal cytogenesis [Du et al., 2001, 2002]. Both NuMA–microtubule and NuMA–LGN interactions are clearly required for normal mitotic progression and disruption of these interactions leads to mitotic delay or arrest. Since the NuMA–microtubule binding domain overlaps with the NuMA–LGN interaction site, NuMA binding to these partners is mutually exclusive [Du et al., 2001]. Although the mechanism by which NuMA binds to LGN in the spindle pole and the cell cortex has been described [Du and Macara, 2004], the machinery that controls the dynamic interactions between NuMA–MTs and NuMA–LGN association is not fully understood. Since the Aurora-A phosphorylation site S1883A/S1884A is adjacent to the NuMA–LGN binging site, we strongly suspect that this phospho-acceptor site may directly or indirectly regulate this dynamic array.

It is well known that Aurora-A is a key kinase for cell survival and an important player in the apoptotic pathway [Qi et al., 2011].

Multiple small molecule Aurora-A kinase inhibitors have been developed and several have progressed to clinical trials. It has been shown that small molecule inhibitors of Aurora-A kinase such as MLN8054 and VX-680 inhibit proliferation of numerous cultured tumor cell lines including PC-3 cells and induce apoptosis in human cultured cells and xenograft tumors in animal models [Harrington et al., 2004; Hoar et al., 2007]. To date, however, the only known Aurora-A phosphorylation site that has been directly linked to apoptosis is the Ser215 site in p53 [Liu et al., 2004]. The dearth of validated physiological substrates has precluded a clear understanding of the mechanisms whereby Aurora-A activity regulates cell death. Here, we demonstrate that Aurora-A phosphorylation of NuMA is important for PC-3 cell survival. Transient transfection of PC-3 cells with NuMA T1804A, T1811A/T1812A, S1887A, S1991A, S2047A, and T2084A/S2087A mutants increased apoptosis up to twofold, however, apoptosis did not increase when cells were transfected with the NuMA T1804A/S2047A double mutant. Although the mechanism whereby this bi-directional second-site suppressor-like effect occurs is not clear, it is tempting to speculate that it may involve changes in the spectrum of protein partners that Aurora-A interacts with.

In addition to a role in apoptosis, our data implicate Aurora-A activity in NuMA localization. NuMA T1804A is unable to fully localize in interphase nuclei and is dispersed throughout the interphase cytoplasm in aggregated particles or clusters. Hyperphosphorylation of NuMA is thought to maintain solubility during mitosis, in concert with the increase in Aurora-A steady-state level during the cell cycle. It is known that solubilized NuMA binds to dynein–dynactin and translocates along with microtubules to the spindle pole [Merdes et al., 2000]. It is likely that phosphorylation of NuMA by Aurora-A at position T1804 affects its solubility, and consequently its transportation and localization to the poles. Additionally, phosphorylation of NuMA at T1804 could potentially affect the interaction between NuMA and its partner protein 4.1R, which co-localizes partially with NuMA in interphase nuclei and at spindle poles [Mattagajasingh et al., 1999]. Through its C-terminal motif, 4.1R binds to NuMA (1,788–1,810) and interacts with the NuMA–dynein–dynactin complex [Mattagajasingh et al., 2009]. NuMA binding to 4.1R is important for proper spindle formation, and depletion of 4.1R from *X. laevis* mitotic extracts leads to NuMA mis-localization, abnormal mitotic spindle formation and mitotic delay [Meyer et al., 2011].

Previous studies have indicated a correlation between Aurora-A overexpression and higher tumor grade as well as poor prognosis in several cancers including prostate cancer, the most frequently diagnosed malignancy in men and second leading cause of cancer death in the United States [Sen et al., 1997; Furukawa et al., 2007; Guan et al., 2007; Brawley, 2012]. Aurora-A overexpression associates with high-grade prostatic intraepithelial neoplasia, indicating that Aurora-A activity may play a role in prostate cancer initiation [Buschhorn et al., 2005; Lee et al., 2006]. Also, Aurora-A expression in biopsy specimens significantly correlates with Gleason score and clinical staging as well as tumor invasion to the seminal vesicle [Lee et al., 2006; Furukawa et al., 2007]. Although phosphorylation and activation of Androgen Receptor by Aurora-A in prostate cancer were reported recently, the pathway/s or exact mechanism by which Aurora-A regulate the apoptotic pathway in prostate cancer and other cancers has not been fully elucidated [Shu et al., 2010]. It is essential to identify Aurora-A substrates that can be used as biomarkers in cancer therapy, and to provide further insights into Aurora-A function. This work identifies NuMA as a physiological Aurora-A substrate, and demonstrates a functional role for Aurora-A phosphorylation in preventing apoptosis.

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