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Nucleophosmin/B23 activates Aurora-A at the centrosome through phosphorylation of Serine 89

Running title: Activation of Aurora-A by Nucleophosmin

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ABSTRACT

Aurora-A (AurA) is a major mitotic protein kinase involved in centrosome maturation and spindle assembly. Nucleophosmin/B23 (NPM) is a pleiotropic nucleolar protein involved in a variety of cellular processes including centrosome maturation. In the present study, we report that NPM is a strong activator of AurA kinase activity. NPM and AurA co-immunoprecipitate and colocalize to centrosomes in G2 phase, where AurA becomes active. In contrast with previously characterized AurA activators, NPM does not trigger autophosphorylation of AurA on Threonine 288 (T288). NPM induces phosphorylation of AurA on Serine 89 (S89) and this phosphorylation is necessary for activation of AurA. These data were confirmed *in vivo* since depletion of NPM by RNA interference eliminated phosphorylation of CDC25B on S353 at the centrosome, indicating a local loss of AurA activity. Our data demonstrate that NPM is a strong activator of AurA kinase activity at the centrosome and support a novel mechanism of activation for AurA.

INTRODUCTION

Mitosis is a complex process that allows the mother cell to divide into two daughter cells. During this event, equal segregation of genetic information is crucial. Indeed, any perturbation during chromosome segregation could lead to aneuploidy, a major cause of cancer (Ganem et al., 2009). The centrosome plays a major role in the cell cycle by serving as a signaling platform and by nucleating mitotic spindle microtubules. The centrosome cycle is regulated concomitantly with cell cycle progression and is controlled by several factors including the mitotic kinase, Aurora-A (AurA). AurA is a serine/threonine kinase that fulfills several key functions during the cell cycle. AurA is involved in G2/M transition, centrosome separation and maturation, mitotic spindle assembly, and in the G2/M and spindle assembly checkpoints. To fulfill these functions, AurA needs to be correctly located and activated at the appropriate time. Several AurA activators have been reported in the past decade, although the data are controversial. Indeed, the first reported AurA activators, Bora and Ajuba, have never been confirmed. Bora is rather an intermediate that stimulates PLK1 phosphorylation by AurA (Seki et al., 2008) and Ajuba has been demonstrated to not activate AurA in *Drosophila* (Sabino et al.). TPX2 (Dodson and Bayliss, 2011; Eyers et al., 2003), HEF1 (Pugacheva and Golemis, 2005), and more recently CEP192 (Joukov et al., 2010) and Arpc1b (Molli et al., 2010) have also been described as AurA activators. All these studies showed an increase in AurA phosphorylation on threonine 288 (T288) concomitant with activation of the kinase. T288 is located in the activation loop of the kinase and is directly involved in the activity of AurA. The fact that AurA is activated sequentially by several molecules suggests a fine tuning of the control of AurA activity and a complex regulatory network where each activator increases the kinase activity for a specific function. It also opens new avenues to discover more activating proteins.

Nucleophosmin/B23 (NPM) is a phosphoprotein localized mainly in the nucleolus where it exerts several of its functions. NPM also localizes to centrosomes and a proportion of the protein continuously shuttles between the nucleus and the cytoplasm. NPM is involved in ribosome biogenesis, centrosome duplication, DNA repair, and response to stress. More recently, NPM was shown to be involved in mitotic spindle formation and regulation of microtubule spindle tension (Amin et al., 2008a; Amin et al., 2008b). NPM has been implicated in the pathogenesis of several human malignancies and described both as an oncogene and a tumor suppressor, depending on the cell type and protein levels.

In the present study, we found that NPM is a strong activator of AurA *in vitro*. Our data show that NPM activates AurA through a novel mechanism that does not depend on T288 phosphorylation. Importantly, we showed that activation strongly depends on auto-phosphorylation of AurA on serine 89 (S89). We found that *in vivo*, AurA and NPM colocalize at the centrosome and coimmunoprecipitate. Activation of AurA by NPM was validated at the cellular level since depletion of NPM by RNA interference leads to a decrease in S353 phosphorylation in CDC25B, a target of AurA at the centrosome.

RESULTS AND DISCUSSION

We initially identified Nucleophosmin/B23 (NPM) in a screen designed to search for AurA substrates in mammalian cell extracts. We evaluated the activity of AurA in an *in vitro* kinase assay using GST-tagged H3-tail as a substrate (Scrittore et al., 2001). Purified histidine-tagged AurA was an active kinase as it phosphorylated H3 (Figure 1A). We also confirmed that AurA phosphorylated GST-tagged NPM *in vitro* (Figure 1B, top panel). Moreover, we detected AurA autophosphorylation (Figure 1A and B). Surprisingly, AurA showed a stronger kinase activity against NPM than H3. An AurA concentration as low as 0.5 pmol was sufficient to phosphorylate NPM, whereas we did not detect any phosphorylation of H3 at that concentration of kinase (data not shown). We only observed phosphorylation of H3 when the kinase concentration was increased up to 20 pmol (Figure 1A). AurA activity is commonly associated with phosphorylation of T288 on AurA (Bischoff et al., 1998). Intriguingly, we did not detect any increase in AurA T288 phosphorylation as revealed by western blot using specific anti-phospho T288 AurA antibody (Figure 1B, bottom panel).

Because AurA kinase activity was more intense when NPM was used as a substrate compared to H3, we investigated whether NPM could act as an activator of AurA kinase activity. We performed enzymatic assays with a limiting amount of kinase (0.5 pmol) (Figure 1C). In the absence of NPM, autophosphorylation of AurA was not detectable and phosphorylation of H3 was hardly detected (Figure 1C, lane 1). The amount of AurA and H3 was kept unchanged and we gradually increased the amount of NPM. As shown in Figure 1C, lanes 3 to 6 in the top panel, addition of increasing amounts of NPM resulted in increased phosphorylation of NPM as well as H3 and AurA. This clearly indicated that stimulation of AurA kinase activity correlated with the amount of NPM protein. Phosphorylation of H3 reached a plateau when the proportion of 5 pmol of NPM for 0.5 pmol of AurA was reached. During the past decade,

several AurA activators were identified. However, mechanisms of activation have only been documented for TPX2 and CEP192. In both cases, activation was associated with a strong phosphorylation of AurA on T288. We observed no significant increase in T288 phosphorylation in AurA as detected with an anti-phospho T288 AurA antibody (Figure 1C), suggesting that NPM could activate AurA through an alternative mechanism. TPX2 activates AurA through conformational changes and triggers protection of the T288 residue from dephosphorylation by PP1 phosphatase (Bayliss et al., 2003; Dodson and Bayliss, 2011). To confirm that NPM does not act like TPX2, we tested whether pre-incubation of AurA with increasing amounts of TPX2, or NPM, protected T288 phosphorylation. As previously reported, TPX2 protected AurA from dephosphorylation by PP1 (Figure 2A). We repeated the experiment with NPM. Addition of increasing amounts of NPM to AurA did not protect T288 from being dephosphorylated by PP1 (Figure 2B). This finding indicates that NPM acts as an AurA kinase activator but, unlike TPX2, does not protect the T288 residue from being dephosphorylated. Even if T288 phosphorylation was not modified when AurA was activated by NPM, this phosphorylation event remained necessary for kinase activation. Indeed, NPM was not able to trigger AurA activation when T287/288 were mutated into alanine, a non phosphorylatable residue (Figure S1). Any AurA protein detected by the anti-phospho T288 antibody is usually considered as active, and this labeling is often used to measure AurA activity (Saskova et al., 2008; Tong et al., 2008). However, three reports suggest that T288 phosphorylation is not an absolute read-out to evaluate AurA activity. First, phosphatase inhibitor-2 (I2) can directly activate AurA without modifying T288 phosphorylation level (Satinover et al, 2004). Second, we showed that activity of *Xenopus laevis* AurA kinase phosphorylated on T295 (equivalent of T288 in human) could be down-regulated during oocyte maturation upon phosphorylation on S349 (Pascreau et al., 2008). Third, Dodson and Bayliss (2011) recently showed that binding of TPX2 without phosphorylation on T288 was

sufficient to activate the kinase.

Figure 1C shows that AurA activation is accompanied by an increase in AurA autophosphorylation. However, Figures 1C and 2B show that T288 is not increasingly phosphorylated in the presence of NPM. These results prompted us to think that phosphorylation of another residue could be involved in the activation. We used phosphoproteomics analysis to determine which residues were phosphorylated in AurA. We found that S89 was the only new residue on AurA to be phosphorylated in the presence of NPM (result obtained from two independent proteomic facilities). S89 is located in the amino terminus extension of AurA and has never previously been reported to be phosphorylated. This part of the kinase likely participates in localization and regulation of the activity of AurA (Rannou et al., 2008). This residue is well conserved among vertebrates. To assess whether S89 phosphorylation could play a role in AurA activation by NPM, we carried out *in vitro* kinase assays with recombinant AurA proteins in which the residue S89 was mutated into an alanine (S89A, which cannot be phosphorylated) and into aspartic or glutamic acid (S89D and S89E respectively, which should mimic a constitutive phosphorylation). We then compared the activity of the AurA S89 mutants with the wild type (WT) kinase by monitoring H3 phosphorylation in presence of increasing amounts of NPM. Figure 2C shows that S89A mutant was not activated by NPM anymore, thus indicating that S89 phosphorylation was necessary for activation of AurA by NPM. S89D or S89E mutants possess a basal activity similar to WT AurA and were not activated by NPM, as S89A mutant. The fact that these mutants did not behave like phosphomimetic mutants was not surprising and it is now well accepted that glutamic or aspartic acid do not systematically mimic phosphorylation (i.e.: (Anthis et al., 2009; Eyers and Maller, 2004; Pascreau et al., 2008). It is likely that similarly to the results obtained by Paleologou et al. (2008), mutation of S89 into glutamic or aspartic acid does not exactly modify AurA conformation as phosphorylation of S89 does. We then

searched for the NPM domain responsible for AurA activation. We used four constructs to produce various truncated NPM proteins (Figure 2D). Figure 2E shows that the domain that allowed activation of AurA was in the C-terminus of NPM, in the P3 fragment. To date, no specific function has been attributed to this NPM domain.

To determine the *in vivo* relevance of the NPM and AurA interaction, we investigated whether the two proteins could colocalize *in vivo*. NPM is a nucleolar protein, also reported to localize to centrosomes (Zatsepina et al., 1995), between centrioles (Okuda et al., 2000). Similarly, AurA localizes to centrosomes from the end of S phase to the beginning of G1 phase (Gopalan et al., 1997; Roghi et al., 1998). We examined the localization of both proteins by confocal microscopy using a rabbit polyclonal anti-NPM antibody and a mouse monoclonal anti-AurA antibody. As expected both proteins were found at the centrosome at the end of interphase as well as in mitosis (Figure 3AB). To study the *in vivo* physical interaction of NPM and AurA, we immunoprecipitated each protein individually from HeLa cells and used western blot to detect the presence of the other protein in the immunoprecipitate. Immunoprecipitations were performed with non-synchronized cells as well as with cells synchronized in mitosis. We obtained the same result in both cases: NPM was detected in AurA immunoprecipitate and AurA was detected in NPM immunoprecipitate (Figure 3C). Therefore, both proteins not only colocalize but also interact *in vivo*.

To investigate whether NPM has any effect on AurA *in vivo*, we examined the phosphorylation of two well-characterized substrates of AurA at the centrosome. First, we monitored PLK1 phosphorylation. This kinase is phosphorylated by AurA on T210 and this event is necessary for mitotic entry (Macurek et al., 2008; Seki et al., 2008). We obtained over 80 % of NPM depletion by transfecting U2OS cells with a plasmid coding a ShRNA (Figure S2A). PLK1 expression level and phosphorylation on T210 were first quantified by western blot. Figure S2A indicates they were not affected by NPM depletion. The lack of modification

in PLK1 phosphorylation status was confirmed by immuno-fluorescence (IF), since PLK1 phospho-T210 centrosomal labeling was not affected in NPM depleted cells (Fig S2B). Next, we examined the effect of NPM depletion on CDC25B phosphorylation. CDC25B is phosphorylated on serine 353 by AurA at the end of G2 just before G2/M transition. This phosphorylation event can be monitored using the phospho-specific SE96 antibody (Cazales et al., 2005; Dutertre et al., 2004; Krystyniak et al., 2006). Under these conditions we analyzed the phosphorylation status of CDC25B on S353 using western blot. We detected a strong decrease in phosphorylation of S353 in CDC25B when NPM was depleted, though the protein levels of CDC25B and AurA were unchanged (Figure 4A). We obtained the same results by using two other ShRNAs targeting different sequences (Figure S3). In contrast with NPM depletion, NPM overexpression (with NPM tagged at the N- or C-terminus end) did not change the phosphorylation of S353 in CDC25B suggesting that, under physiological conditions, AurA is likely to be fully activated by NPM (data not shown). We further asked *in vitro* whether NPM was able to activate phosphorylation of CDC25B by AurA similarly to what we observed concerning H3. Figure 4B confirmed that addition of increasing amounts of NPM activated the kinase and increased phosphorylation of CDC25B. To verify that lack of S353 phosphorylation of CDC25B in the absence of NPM was occurring at the centrosome, we used the same anti-phospho-CDC25B antibody to evaluate the phosphorylation of S353 in proliferating cells by immunofluorescence. Late G2 cells were distributed according to the intensity of centrosomal labeling. In control cells, about 10% of cells showed no labeling, 45% showed weak labeling, and about 45% of cells showed strong labeling (Figure 4C). When NPM was depleted, 35% of cells showed no labeling, 50% showed weak labeling, and only 15% of cells showed strong labeling (Figure 4D). These results clearly indicated a decrease in CDC25B phosphorylation on S353 in the absence of NPM, demonstrating that AurA kinase was less active at the centrosome in the absence of NPM. They also indicated that activation

of AurA by NPM was quite specific towards CDC25B since PLK1 phosphorylation was not affected by NPM depletion. This last result could also be explained by the fact that phosphorylation of PLK1 by AurA is already stimulated by Bora (Macurek et al., 2008; Seki et al., 2008). Importantly, similar to our *in vitro* findings, phosphorylation of AurA on T288 was not dependent on the presence of NPM (Figure 4E).

We then wanted to confirm *in vivo* that AurA activation by NPM acts through phosphorylation of S89 on AurA. Due to lack of S89 phospho-specific antibody we could not directly evaluate the phosphorylation state of S89 in presence or absence of NPM. To study the effect of S89 phosphorylation on AurA activity *in vivo*, we examined the effect of expression of S89 mutant versions of Aurora-A on CDC25B phospho-S353 labeling at the centrosome. Cells were first transfected with plasmids encoding for WT, S89A, S89D, or S89E GFP-tagged AurA proteins, resulting in physiological level of expression of the WT or mutated kinases. Endogenous AurA was depleted by transfection with a SiRNA that does not target the transfected versions of the kinase. Figure 5A indicates that, similarly to NPM depletion, AurA depletion triggered an increase in the number of cells with centrosomes that showed no CDC25B phospho-S353 labeling and a decrease in the number of cells presenting a strong CDC25B phospho-S353 labeling. This phenotype was rescued by WT AurA but not by S89A AurA, thus suggesting that phosphorylation of AurA on S89 is necessary for the kinase to be activated by NPM. In agreement with our previous biochemical data showing that S89D and S89E mutations were not phosphomimetic, S89D and S89E mutants could not rescue AurA depletion.

AurA plays many crucial roles in cell cycle regulation and mitosis achievement. Notably, AurA was proven to be involved in microtubules growth through recruitment of microtubules nucleating factors at the centrosomes (For review, see Joukov (2011)). NPM also plays key roles in centrosome biology. It has recently been shown to be involved in mitotic spindle

formation and regulation of spindle microtubule nucleation and tension (Amin et al., 2008a; Amin et al., 2008b). NPM involvement in microtubules dynamics could act through its interaction with AurA. To test this hypothesis, we examined the effect of NPM or AurA depletion on microtubules nucleation. Cells were treated by nocodazole 33 μ M for 3 hours to depolymerize microtubules and then washed four times with pre-warmed fresh medium. Two minutes after washes, cells were fixed and labeled for α -tubulin and γ -tubulin, then microtubules nucleating from centrosomes in G2 phase cells were measured. Figure 5B showed that microtubules were shorter in cells depleted for NPM or AurA than in control. WT AurA was able to rescue this phenotype, contrary to S89A AurA. This suggested that phosphorylation of AurA on S89 is involved in microtubules nucleation and that NPM likely participates in nucleation through activation of AurA. It is particularly interesting to notice that CDC25B, like NPM, is also involved in centrosome duplication (Boutros et al., 2007) and mitotic spindle formation through microtubule nucleation (Gabrielli et al., 1996). Our study thus suggests a connection between two independent pathways implicating (i) Cyclin E/Cdk2, NPM, or ROCK II and (ii) AurA, CDC25B, or Centrin 2 and could open up new perspectives concerning centrosome duplication and maturation studies.

MATERIALS AND METHODS

DNA constructs and protein purification

pGEX-B23 was provided by K. Fukazawa. pGEX-4T3-NPM-P1, P2, P3, P4 were a kind gift from I. Hoffmann. pMAL-C2-CDC25B3 was a kind gift of Bernard Ducommun. AurA and H3-tail cDNA were cloned in pET29 and pGEX vectors respectively. AurA mutants (S89A, S89D, S89E, T287/288A) were generated with the QuickChange mutagenesis kit (Stratagene). AurA depletion in U2OS cells was achieved as described in (Dutertre et al., 2004). NPM depletion in U2OS cells was achieved after transfection with PLKO plasmids (Ref NM_002520.4-169s1c1, Sigma) targeting the following sequences: #1, CCGGGCCGACAAAGATTATCACTTTCTCGAGAAAGTGATAATCTTTGTCGGCTTT, #2, CCGGGCAAAGGATGAGTTGCACATTCTCGAGAATGTGCAACTCATCCTTTGCTTT TTG or #3, CCGGCCTAGTTCTGTAGAAGACATTCTCGAGAATGTCTTCTACAGAACTAGGTTTT TG. Luciferase ShRNA was used as control. AurA WT and S89 mutant versions were cloned in a pEGFP-C1 vector with the AurA promoter sequence to allow physiological expression level. For recombinant proteins production, BL21 *Escherichia Coli* were transformed with plasmids, induced by 1 mM IPTG for 4h, pelleted and frozen at -80°C. Purification of histidine-tagged proteins (human AurA, Histone H3) was performed as previously described (Roghi et al., 1998). Briefly, bacterial pellets were lysed and loaded onto Ni-NTA-agarose beads (Qiagen), washed and proteins were eluted with buffer containing imidazole. Purification of GST-tagged proteins (human NPM and NPM fragments) was performed with Gluthatione Sepharose 4B (Amersham) in accordance with the manufacturer's instructions. Pellets were lysed and loaded into Gluthatione Sepharose 4B (Amersham). MBP-CDC25B3

was produced in JM109 bacteria and affinity purified on amylose beads following the manufacturer's instructions (New England Biolabs). Purified proteins were stored at -80°C or in 50% glycerol at -20°C.

Cell lines and transfection

Human HeLa cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with penicillin and streptomycin (Invitrogen) and 10% fetal calf serum (PAA). Human U2O2 cells were grown in Mc Coy's Medium with penicillin and streptomycin (Invitrogen) and 10% fetal calf serum (PAA). Cells were transfected in culture medium using Jetprime (Polyplus Transfection) in accordance with the manufacturer's instructions. Media were changed after 24h. Mitotic cells were collected by mitotic shake off after incubation in 1 µg/ml Taxol for 16 h. For expression of GFP-AurA WT or S89 mutant versions, cells were transfected for 24h hours with the appropriate plasmid and then, endogenous AurA was depleted by transfection with SiRNA for 24h.

Immunofluorescence

Cells were preliminarily plated onto glass coverslips. The cells were then washed in cold PBS with calcium, fixed with methanol at -20°C for 5 min (3 minutes for CDC25B phospho-S353 and CDC25B), and then washed three times in cold PBS and saturated with PBS-BSA 5% (PBS-SVF 5% for CDC25B phospho-S353 and CDC25B) for 1 h at room temperature. Antibodies in PBS-BSA 5% (PBS-SVF 5% for CDC25B phospho-S353 and CDC25B) were added onto the cells: rabbit anti-NPM PabN1 1:100 (Shinmura *et al.*, 2005); mouse anti-gamma-tubuline 1:500 (clone GTU88, Sigma); mouse anti-AurA (clone 35C11:20) (Cremet *et al.*, 2003); CDC25B and CDC25B phospho-S353, 1:100, (gift from B. Ducommun); PLK1 (Zymed) and PLK1 phospho-T210 (BD Pharmingen), 1:1000. These were left overnight at

4°C, and then washed 3 times and incubated in the dark with secondary antibodies (anti-Mouse-; anti-Rabbit-488 or 555 1:1000, Alexa, Invitrogen) for 1 h at room temperature. After 3 final washes with PBS, coverslips were mounted with ProlongGold (Invitrogen) with 1 µg/mL DAPI (Sigma). Cells were examined using a Leica DMRXA2 fluorescent microscope with a X63 oil immersion objective. Images were processed using Metamorph software (Universal Imaging). Cells were also observed with a confocal SP2 microscope (Leica) and images were processed with Leica software.

Western blot analysis

Cells were lysed in RIPA buffer containing anti-protease (Complete, Roche) and lysates were clarified by centrifugation (13000 rpm, 30 min, 4°C). Proteins were assayed by Bradford assay (BioRad). Equal amounts of protein lysates in Laemmli were loaded onto 12.5% SDS-PAGE gel for electrophoresis and transferred onto nitrocellulose membranes. Membranes were blocked for 1 h at room temperature with TBST- 4% milk (5% BSA for NPM), and then incubated overnight with the following antibodies: Mouse anti-β-tubuline (1:2000), Mouse anti-NPM (Sigma, 1:2000), mouse anti-AurA (clone 35C1 1:100) (Cremet et al., 2003); rabbit anti-CDC25B and CDC25B phospho-S353 (1:1000) (gift from B. Ducommun), mouse anti-PLK1 (Zymed) and PLK1 phospho-T210 (BD Pharmingen). Secondary antibodies coupled with HRP (Jackson) were incubated for 1 h and antibody binding was detected by enhanced chemiluminescence (Pico or Dura, Pierce).

In vitro kinase assays

Different amounts of recombinant AurA, recombinant nucleophosmin, and recombinant H3 or CDC25B—as stated in the figures— were mixed in 50 mM Tris HCl pH 7.5, 25 mM NaCl, 1 mM DTT, 0.01% TritonX-100, 10 mM MgCl₂. Then, 100 µM ATP and 2 µCi of [γ -³²P]

ATP were added to the reaction mixture and incubated at 37°C for 10 min. Reactions were stopped in Laemmli buffer, boiled for 5 min, and resolved on a 12.5% SDS-PAGE gel. Gels were either stained with Coomassie blue, fixed, dried and autoradiographed with Storm 840 (Molecular Dynamics), or were transferred onto nitrocellulose membrane, autoradiographed for 3 days on X-Omat films (Kodak), and then blocked with PBS-4% milk and subjected to immunoblots.

Mass spectrometry analysis

AurA was proven to be phosphorylated even when purified from *E. coli* as a recombinant protein (Haydon et al., 2003). To clearly discriminate between phosphorylation events, recombinant AurA (1 µg) was incubated 20 minutes at 37°C, either alone, or in presence of 20 mM ATP, or in presence of 20 mM ATP and 1µg NPM. Enzymatic reactions were stopped with Laemmli buffer. Proteins were separated in SDS-PAGE and the gel was stained with Coomassie blue.

The gel slices were destained with 50% methanol then reduced in 10 mM DTT for 1 hour at 56°C and alkylated in 55mM chloroacetamide for one hour at room temperature. After washing in 50 mM Ammonium Bicarbonate, the gel pieces were shrunk in 100% acetonitrile. The digestion was performed using Trypsin in 50 mM Ammonium Bicarbonate for 8 hours at 37°C and 600 rpm. The peptides were finally extracted in 90% ACN/0.5M Urea and dried in Speed Vac. Samples were resolubilized in ACN 5% formic acid (FA) 0.2%. Samples were separated on a home-made C18 column (150 µm × 10 cm) using an Eksigent nanoLC-2D system. A 56-min gradient from 5–60% acetonitrile (0.2% FA) was used to elute peptides from a homemade reversed-phase column (150 µm i.d. x 100 mm) with a flow rate set at 600 nanoliter/min.

The column was directly connected to a nanoprobe interfaced with an LTQ-Orbitrap Velos mass spectrometer from Thermo-Fisher. Each full MS spectrum was followed by twelve MS/MS spectra (thirteen scan events), where the twelve most abundant multiply charged ions were selected for MS/MS sequencing. Tandem MS experiments were performed using collision-induced dissociation in the linear ion trap. The data were processed using Mascot 2.1 (Matrix Science) search engine with tolerance parameters set to 15 ppm and 0.5 Da for the precursor and the fragment ions respectively. The selected variable modifications were carbamidomethyl (C), deamidation (NQ), oxidation (M) and phosphorylation (STY). The selected database was human IPI database v.3.54 with 150 858 sequences.

Immunoprecipitation

ProteinG Sepharose Fast Flow (Amersham) and monoclonal anti-AurA (Cremet et al., 2003) clone 35C1 or monoclonal anti-B23 (Sigma) were incubated overnight at 4°C, and then washed extensively. Cells were lysed in 20 mM EDTA, 15 mM MgCl₂, 20 mM Hepes pH7.5, 0.1% Triton, antiprotease (Complete, Roche) and anti-phosphatase cocktail (50 mM NaF, 80 mM beta-glycerophosphate, 1 mM Na₃VO₄). Cell lysates were loaded onto ProteinG Sepharose Fast Flow overnight at 4°C, then subjected to extensive washes in lysis buffer for 4h at 4°C. Samples were finally eluted in Laemmli and boiled for 5min at 90°C or used in a kinase assay.

Microtubules nucleation assay

Cells were incubated 3 hours with 33 μM nocadazole in culture medium. Then the medium was removed and cells were washed four times with fresh pre-warmed medium. Two minutes after last wash, cells were fixed with cold methanol and then immuno-labeled for γ- and β-tubulin. Length of microtubules was measured with the Metamorph software.

Online supplemental material

Figure S1 indicates that NPM was not able to activate a T287/288A AurA mutant. Figure S2 shows that NPM depletion did not affect phosphorylation of PLK1. Figure S3 shows that three different ShRNA designed to target NPM triggered a decrease in CDC25B phosphorylation.

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FIGURE LEGENDS**Figure 1**

Aurora-A phosphorylates H3 and NPM *in vitro*. (A) 20 pmol of AurA was incubated 10 min at 37°C with 10 pmol of H3. H3 phosphorylation was assessed by autoradiography (upper panel) and the presence of AurA and H3 proteins were verified by Coomassie blue staining (lower panel). (B) 0.5 pmol of AurA was incubated 10 min at 37°C with 10 pmol of NPM. NPM phosphorylation was assessed by autoradiography (upper panel) and the presence of NPM protein was assessed by Coomassie blue staining (middle panel). Autophosphorylation of AurA on T288 was verified by western blot (lower panel). (C) 0.5 pmol of AurA was incubated with 10 pmol H3 and the indicated amount of NPM. NPM, AurA, and H3 phosphorylation were assessed by autoradiography (upper panel). NPM, H3, AurA, and AurA autophosphorylation on T288 were evaluated by western blot or Coomassie blue staining.

Figure 2

NPM activates Aurora-A through S89 phosphorylation without protecting T288 from dephosphorylation. (A and B) 0.5 pmol of AurA was incubated with the indicated amount of TPX2 or NPM and then treated with 0.05 U of PP1. AurA, NPM proteins, and AurA autophosphorylation on T288 were evaluated by western blot. (C) 0.5 pmol of WT AurA, or the indicated mutant, was incubated with 10 pmol of H3 and the indicated amount of NPM. H3 phosphorylation was quantified by autoradiography. (D) Scheme representing the different fragments of NPM used in (E). (E) 0.5 pmol of AurA was incubated with 10 pmol H3 and the indicated amounts of the fragments of NPM presented in (D). H3 phosphorylation was evaluated by autoradiography.

Figure 3

NPM and Aurora-A colocalize from the end of G2 to M phase and interact *in vivo*. IF microscopy of cells in G2 phase (A) or metaphase (B). The small upper panel is an enlargement of the centrosome area (scale bar represents 1 μm). NPM appears in red, GFP-AurA in green, and DNA in blue. (C) Western blot showing coimmunoprecipitation of AurA and NPM from HeLa cells protein extract.

Figure 4

NPM depletion triggers a decrease in CDC25B phosphorylation. (A) Protein lysates prepared from control or NPM-depleted U2OS cells were immunoblotted with indicated antibodies. (B) 0.5 pmol of AurA was incubated with 10 pmol of CDC25B and the indicated amount of NPM. CDC25B phosphorylation was quantified by autoradiography. (C) IF characterization of CDC25B phospho-S353 labeling in control or NPM depleted U2OS cells. Control or NPM depleted cells were classified into three categories (no, weak or strong labeling) according to the intensity of CDC25B phospho-S353 labeling. (D) Histogram representing distribution of control or NPM depleted cells in each category. Values are means of three independent experiments. Fifty cells were counted for each experiment. Error bars represent standard error. (E) Protein lysates prepared from control or NPM-depleted U2OS cells were immunoblotted with anti-NPM, anti-AurA, anti-AurA phospho-T288 and with anti- β -tubulin as a loading control.

Figure 5

Aurora-A S89 phosphorylation is required for CDC25B phosphorylation and microtubules nucleation at centrosomes (A) Expression of AurA in U2OS cells transfected

with indicated SiRNA and/or plasmids was verified by western blot (upper panel). Anti- β -tubulin serves as a loading control. For each corresponding cell population, the intensity of centrosomal CDC25B phospho-S353 labeling was evaluated and cell were classified into three categories (no, weak or strong labeling). Data are summarized in the histogram (lower panel). Values are means of three independent experiments. Fifty cells were counted for each experiment. Error bars represent standard error. (B) Immuno-fluorescence microscopy of cells with nucleating microtubules at centrosomes. Length of microtubules is summarized in histogram. Data are representative of three independent experiments. Error bars represent standard error.

Supplemental material

Figure S1

NPM is not able to activate a T287/288A mutant of Aurora-A. 0.5 pmol of T287/288A AurA was incubated with 10 pmol of H3 and the indicated amount of NPM. H3 phosphorylation was quantified by autoradiography (Upper panel). NPM and H3 presence were assessed by Coomassie blue staining (Lower panel).

Figure S2

NPM depletion does not trigger modification in PLK1 phosphorylation status (A) The lysates prepared from control or NPM depleted U2OS cells were immunoblotted with indicated antibodies. (B) Immuno-staining characterization of PLK1 phospho-T210 labeling in control or NPM-depleted U2OS cells. (C) Histogram representing the fluorescence intensity of PLK1 phospho-T210 labeling at the centrosome of control or NPM depleted cells. Values are representative of three independent experiments. Twenty cells were counted for each experiment. Error bars represent standard error.

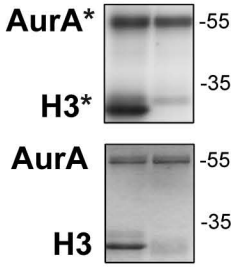
Figure S3

Depletion of NPM by use of two other ShRNA triggers a decrease in CDC25B phosphorylation on S353.

A

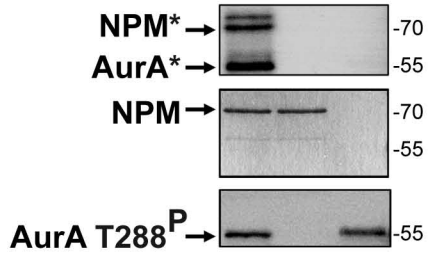
AurA (20 pmol)	+	+
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H3 (10 pmol)	+	-
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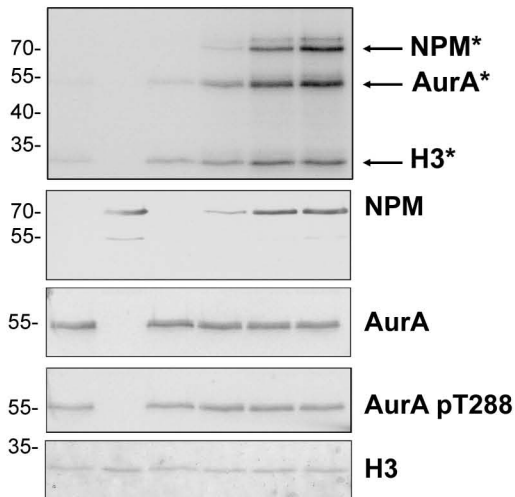
**B**

AurA (0.5 pmol)	+	-	+
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NPM (10 pmol)	+	+	-
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**C**

H3 (10 pmol)	+	+	+	+	+	+
AurA (0.5 pmol)	+	-	+	+	+	+
NPM (pmol)	0	7.5	0.25	2.5	5	7.5

**Fig 1ABC**

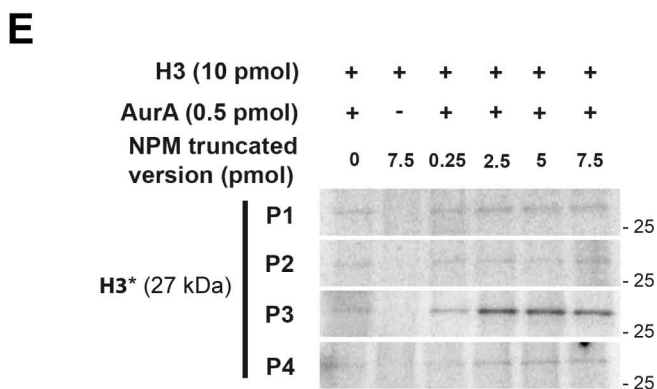
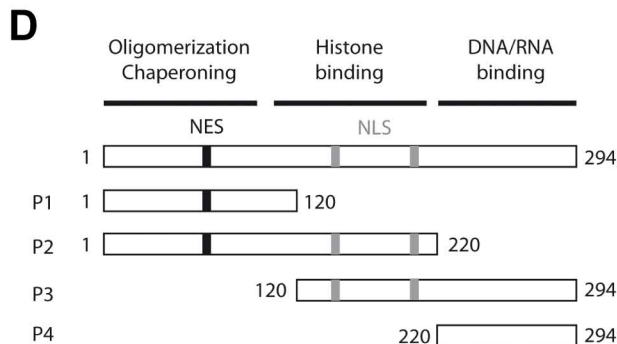
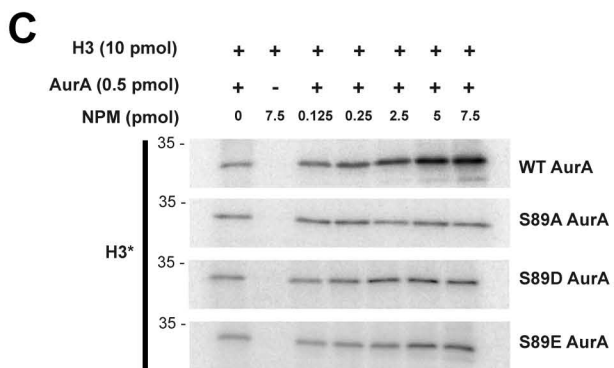
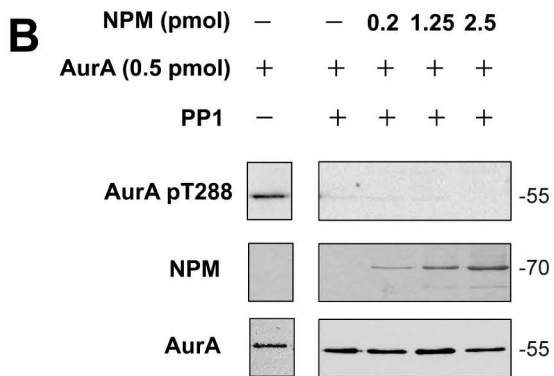
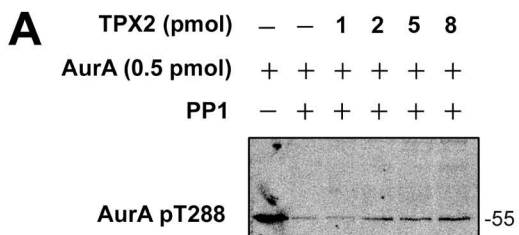


Fig 2ABCDE

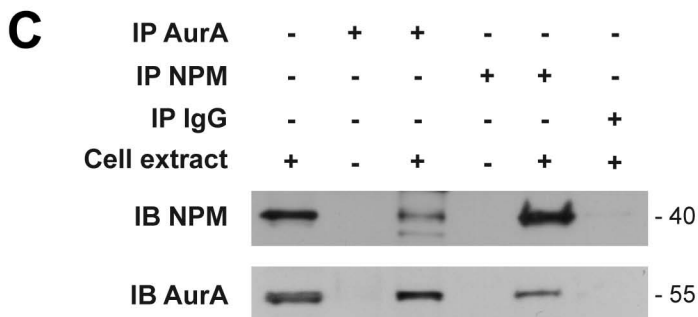
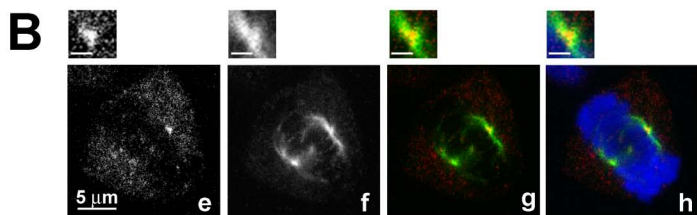
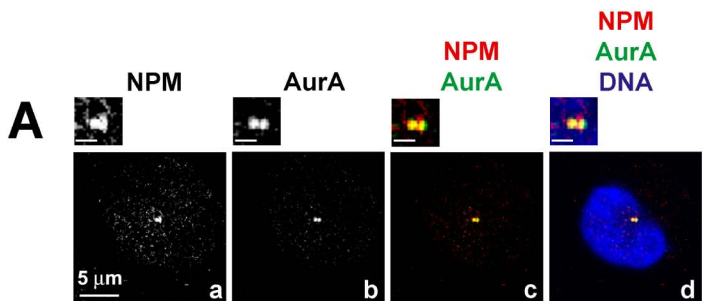


Fig 3ABC

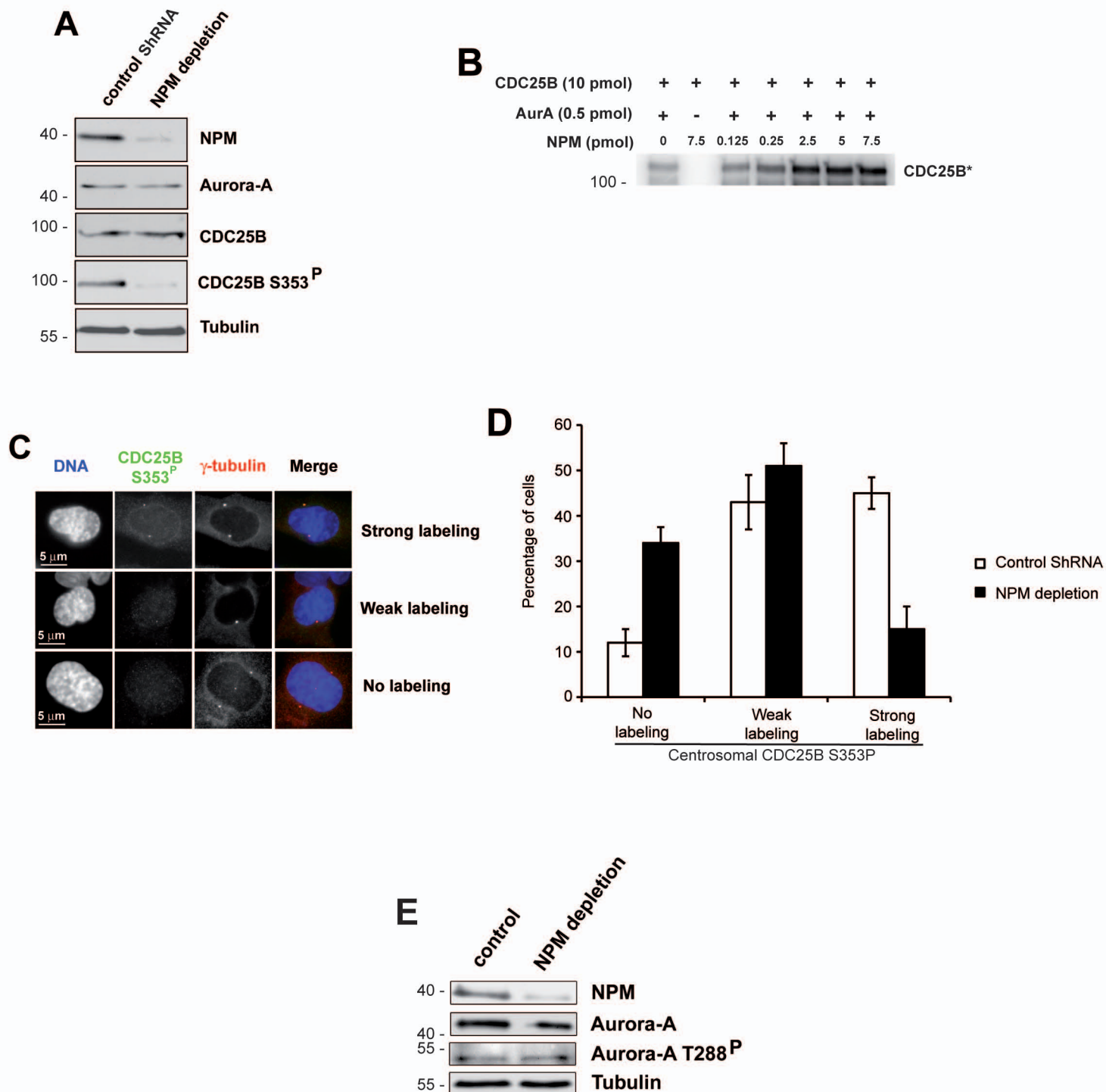
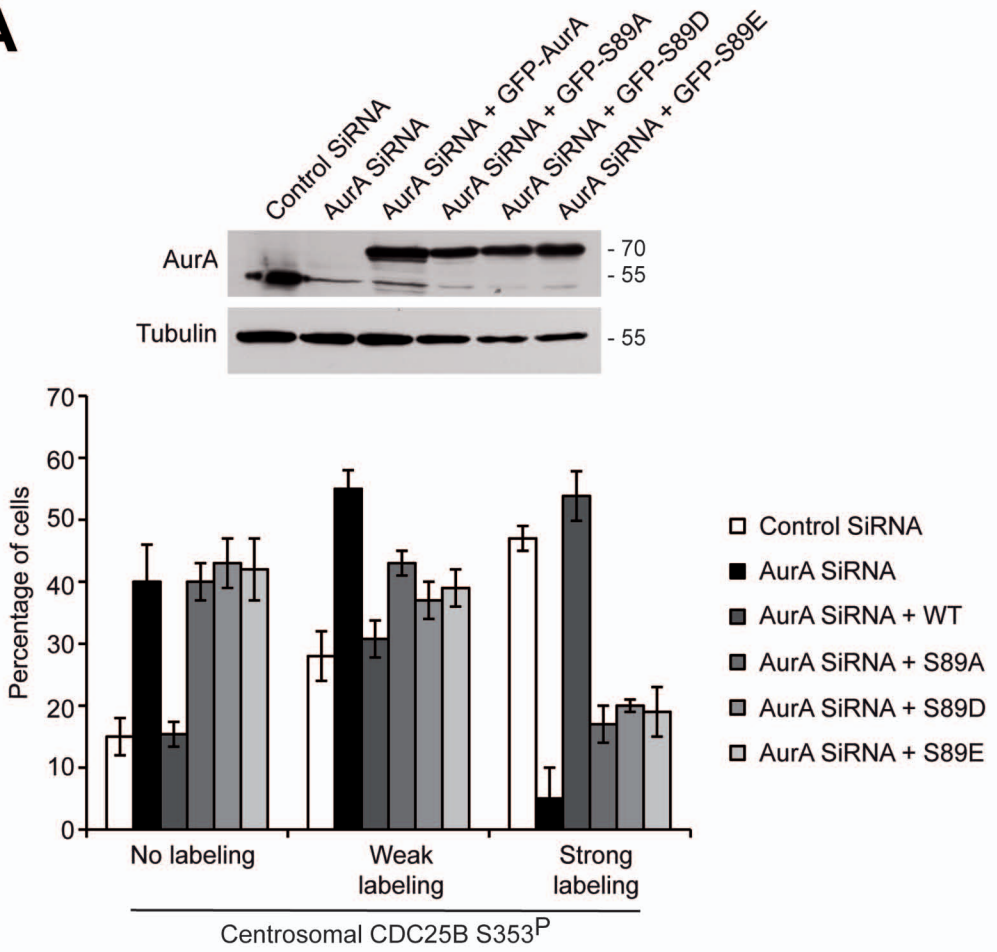
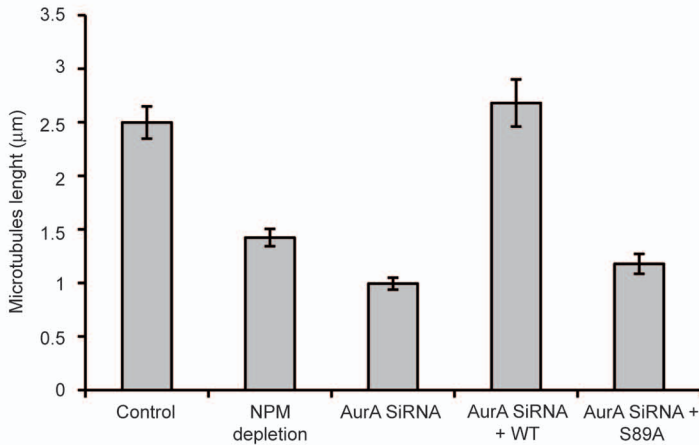
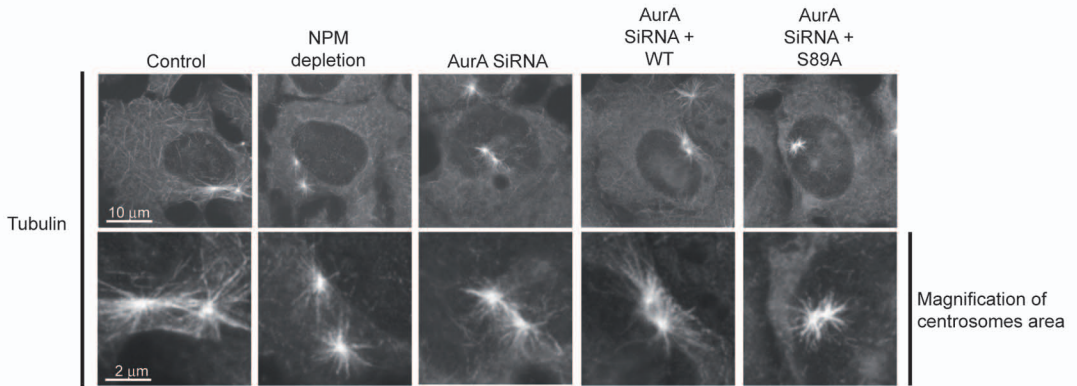


Fig 4ABCDE

A**B****Fig 5AB**

H3 (10 pmol)	+	+	+	+	+	+	+
AurA T287/288A (0.5 pmol)	+	-	+	+	+	+	+
NPM (pmol)	0	7.5	0.125	0.25	2.5	5	7.5



Figure S1

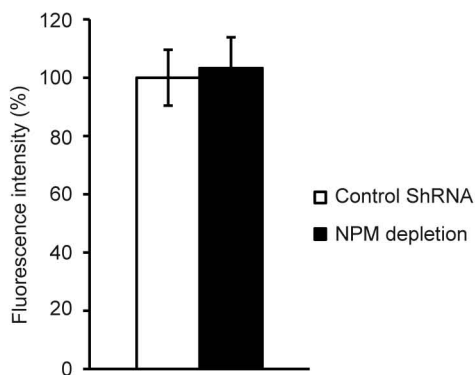
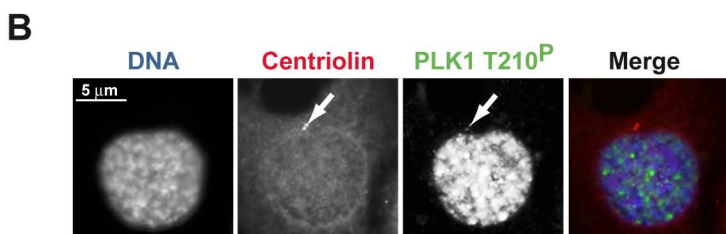
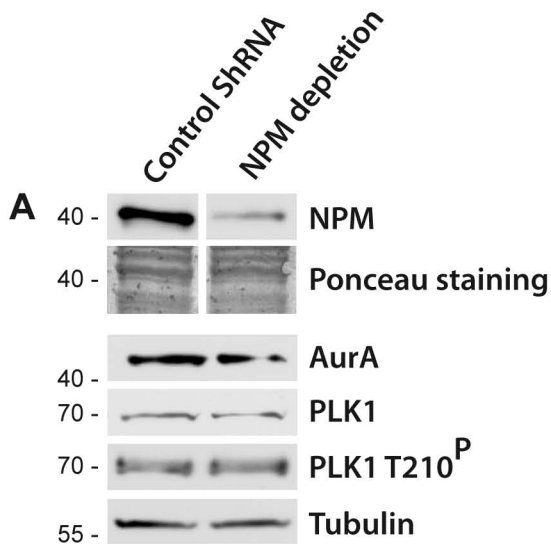


Fig S2AB

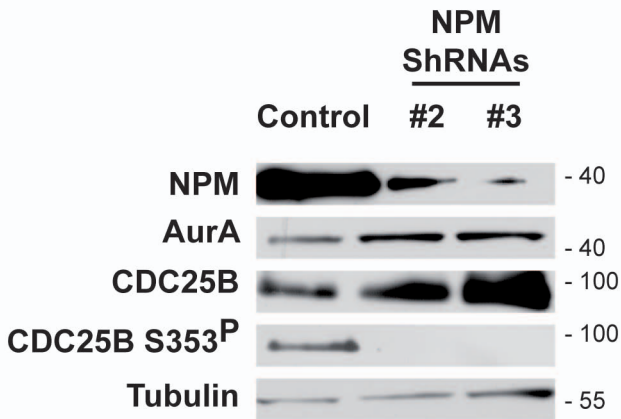


Fig S3