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Human TPX2 is required for targeting Aurora-A kinase to the spindle

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Aurora-A is a serine-threonine kinase implicated in the assembly and maintenance of the mitotic spindle. Here we show that human Aurora-A binds to TPX2, a prominent component of the spindle apparatus. TPX2 was identified by mass spectrometry as a major protein coimmunoprecipitating specifically with Aurora-A from mitotic HeLa cell extracts. Conversely, Aurora-A could be detected in TPX2 immunoprecipitates. This indicates that subpopulations of these two proteins undergo complex formation *in vivo*. Binding studies demonstrated that the NH₂ terminus of TPX2 can directly interact with the

COOH-terminal catalytic domain of Aurora-A. Although kinase activity was not required for this interaction, TPX2 was readily phosphorylated by Aurora-A. Upon siRNA-mediated elimination of TPX2 from cells, the association of Aurora-A with the spindle microtubules was abolished, although its association with spindle poles was unaffected. Conversely, depletion of Aurora-A by siRNA had no detectable influence on the localization of TPX2. We propose that human TPX2 is required for targeting Aurora-A kinase to the spindle apparatus. In turn, Aurora-A might regulate the function of TPX2 during spindle assembly.

Introduction

One of the most critical steps during cell cycle progression is the correct segregation of sister chromatids during mitosis. This process depends on the reorganization of the interphase microtubules (MTs)* into a highly dynamic bipolar array known as the mitotic spindle. In animal cells, spindle assembly is initiated at the time when centrosomes separate and involves changes in the functional properties of several MT-associated proteins (Karsenti and Vernos, 2001; Wittmann et al., 2001). In many cases, these changes are brought about by reversible phosphorylation, and several protein kinases and phosphatases undergo at least transient association with either centrosomes or spindle MTs (Nigg, 2001). However, little is known about the mechanisms that underlie the recruitment of these enzymes to the spindle apparatus, let alone their exact function and regulation during spindle assembly.

Prominent among the kinases implicated in the regulation of spindle-associated events during early mitosis is Aurora-A, a member of the Aurora family of serine-threonine kinases (Bischoff and Plowman, 1999; Nigg, 2001). Studies in several

animal species concur to demonstrate that Aurora-A localizes to centrosomes and the spindle and that protein and activity levels peak at the G2/M transition (Bischoff and Plowman, 1999; Giet and Prigent, 1999). In *Drosophila*, the disruption of Aurora-A function causes centrosome disorganization and a reduction in the length of astral MTs (Glover et al., 1995; Giet et al., 2002). These phenotypes may relate to the ability of Aurora-A to interact with and phosphorylate D-TACC, a centrosome-associated protein implicated in the regulation of MT dynamics (Giet et al., 2002). Similarly, when Aurora-A (AIR-1) was eliminated by RNA-mediated interferences from *Caenorhabditis elegans* embryos, spindle assembly failed, and centrosome maturation, a process characterized by the recruitment of γ -tubulin and other components of the pericentriolar material, did not occur (Hannak et al., 2001). Biochemical studies performed in *Xenopus* egg extracts confirm an important role of Aurora-A in spindle assembly and/or maintenance (Roghi et al., 1998; Giet and Prigent, 2000). In particular, the kinesin-related motor protein Eg5 was identified as a candidate substrate of Aurora-A (Giet et al., 1999). In human cells, overexpression of Aurora-A was found to interfere with mitotic exit, causing tetraploidization and concomitant amplification of centrosomes (Meraldi et al., 2002). Furthermore, overexpression of Aurora-A resulted in neoplastic transformation of NIH-3T3 cells (Zhou et al., 1998). This is intriguing, since Aurora-A is frequently overexpressed in human tumors and the Aurora-A gene maps to a chromosomal locus

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*Abbreviations used in this paper: IVT, *in vitro* transcription/translation; KD, catalytically inactive; MT, microtubule; WT, wild-type.

Key words: spindle; mitosis; Aurora-A; TPX2; siRNA

(20q13.2–13.3) that is often amplified in colon, breast, and stomach cancers (Sen et al., 1997).

To better understand the role of Aurora-A in the regulation of spindle assembly and mitotic progression in human cells, we used a biochemical approach to search for proteins that specifically interact with Aurora-A. We report the identification of the human homologue of the spindle component TPX2 as a novel interactor and substrate of Aurora-A.

Results and discussion

Identification of a TPX2–Aurora-A complex in mitotic cells

To search for proteins interacting with Aurora-A, an antibody raised against a COOH-terminal peptide was used to immunoprecipitate Aurora-A from mitotic HeLa cell extracts. As shown by SDS-PAGE and Coomassie blue staining, the Aurora-A immunoprecipitate contained a protein of the expected size (45 kD) and additional proteins migrating at ~100 kD and >205 kD, respectively (Fig. 1 A, lane 2). None of these proteins were present when immunoprecipitations were performed with beads only (Fig. 1 A, lane 1) or in the presence of excess competing peptide (Fig. 1 A, lane 3). Mass spectrometry confirmed the identity of the 45 kD protein as Aurora-A (unpublished data). More importantly, it identified the 100-kD band as the human homologue of TPX2. By the use of a monoclonal antibody (Ki-S2), this protein had been shown previously to associate with the mitotic spindle (Heidebrecht et al., 1997). Furthermore, *Xenopus* TPX2 has been implicated in targeting the kinesin-related motor XKlp2 to the spindle (Wittmann et al., 1998) and has been shown to be essential for the formation of a robust bipolar spindle (Wittmann et al., 2000). Most recently, TPX2 was also identified as a critical component involved in promoting the RanGTP-regulated nucleation of MTs in the vicinity of chromosomes (Gruss et al., 2001).

To obtain information about the efficiency of complex formation between TPX2 and Aurora-A in mitotic cells, increasing amounts of Aurora-A immunoprecipitates were probed for the presence of TPX2 and vice versa (Fig. 1 B). The two proteins could readily be coimmunoprecipitated, regardless of whether anti-Aurora-A or anti-TPX2 antibodies were used for immunoprecipitation. No spurious precipitations were observed upon blocking the Aurora-A antibody with excess antigenic peptide or when using beads alone for control (Fig. 1 B, lanes 5). The comparison with the Western blot signals seen in total cell lysates (Fig. 1 B, lanes 1) indicates that, under the conditions used here, only a fraction (<5%) of TPX2 is recovered in a complex with Aurora-A and vice versa. What proportions of the two proteins associate *in vivo* is difficult to estimate, since the recovery of the complex is expected to depend on solubilization conditions. In any event, it appears likely that the *in vivo* formation of the TPX2–Aurora-A complex is regulated in a dynamic fashion.

TPX2–Aurora-A interaction is direct and independent of kinase activity

To determine whether the binding of TPX2 to Aurora-A depends on its kinase activity, GST-tagged constructs of wild-

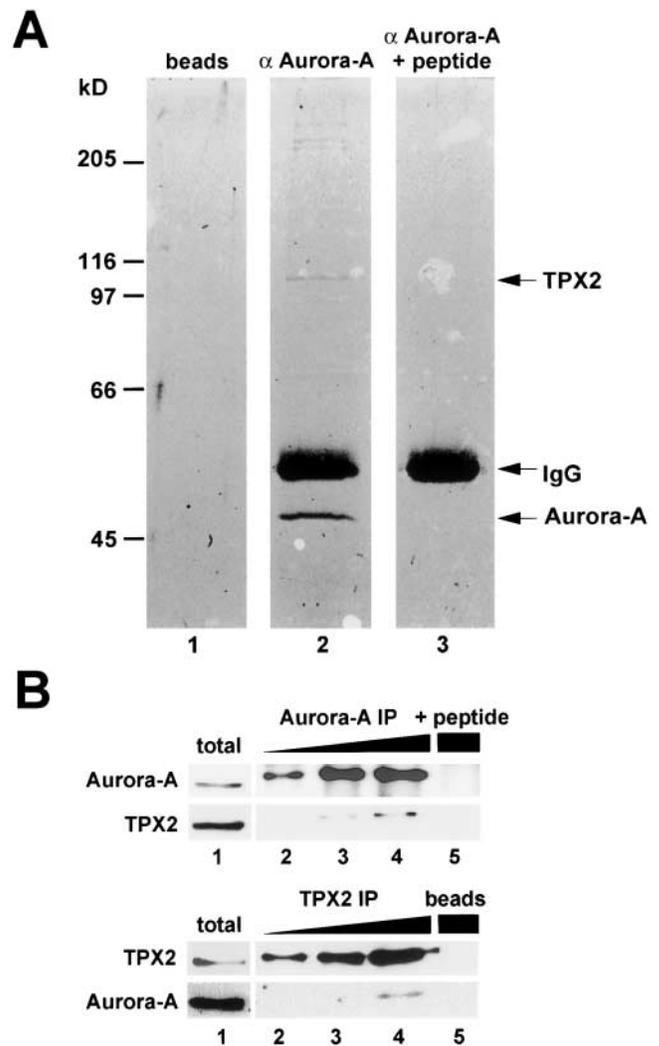


Figure 1. Identification of TPX2 as an Aurora-A-interacting protein. (A) Coomassie blue-stained gel of Aurora-A immunoprecipitate from mitotic HeLa cell extract (lane 2). For control, immunoprecipitations were also performed with either protein A-coated beads alone (lane 1) or Aurora-A antibody blocked with an excess of antigenic peptide (lane 3). Arrows point to the precipitated 45-kD Aurora-A protein, IgG heavy chain, and the 100-kD protein identified as TPX2 by tryptic peptide fingerprinting. The identity of the high molecular weight proteins coprecipitating with Aurora-A is currently under investigation. (B, top) Increasing amounts of Aurora-A immunoprecipitates (lanes 2–4) were separated by SDS-PAGE and analyzed by Western blotting using antibodies against Aurora-A and TPX2. The control immunoprecipitation was performed with the peptide blocked Aurora-A antibody (lane 5). (B, bottom) TPX2 coimmunoprecipitates were analyzed by Western blotting using antibodies against Aurora-A and TPX2. Beads alone were used for the control precipitation (lane 5). Total cell lysates were analyzed in parallel (lane 1).

type (WT) and catalytically inactive (KD) recombinant Aurora-A kinases were immobilized on glutathione Sepharose and incubated with extracts prepared from mitotic HeLa cells. Proteins bound to Aurora-A were then analyzed by SDS-PAGE and Western blotting (Fig. 2 A). Coomassie blue staining (Fig. 2 A, top) revealed a prominent band migrating at 100 kD as the major interaction partner of Aurora-A (Fig. 2 A, top, arrowheads). This protein was identified as TPX2 by both Western blotting (Fig. 2 A, bottom)

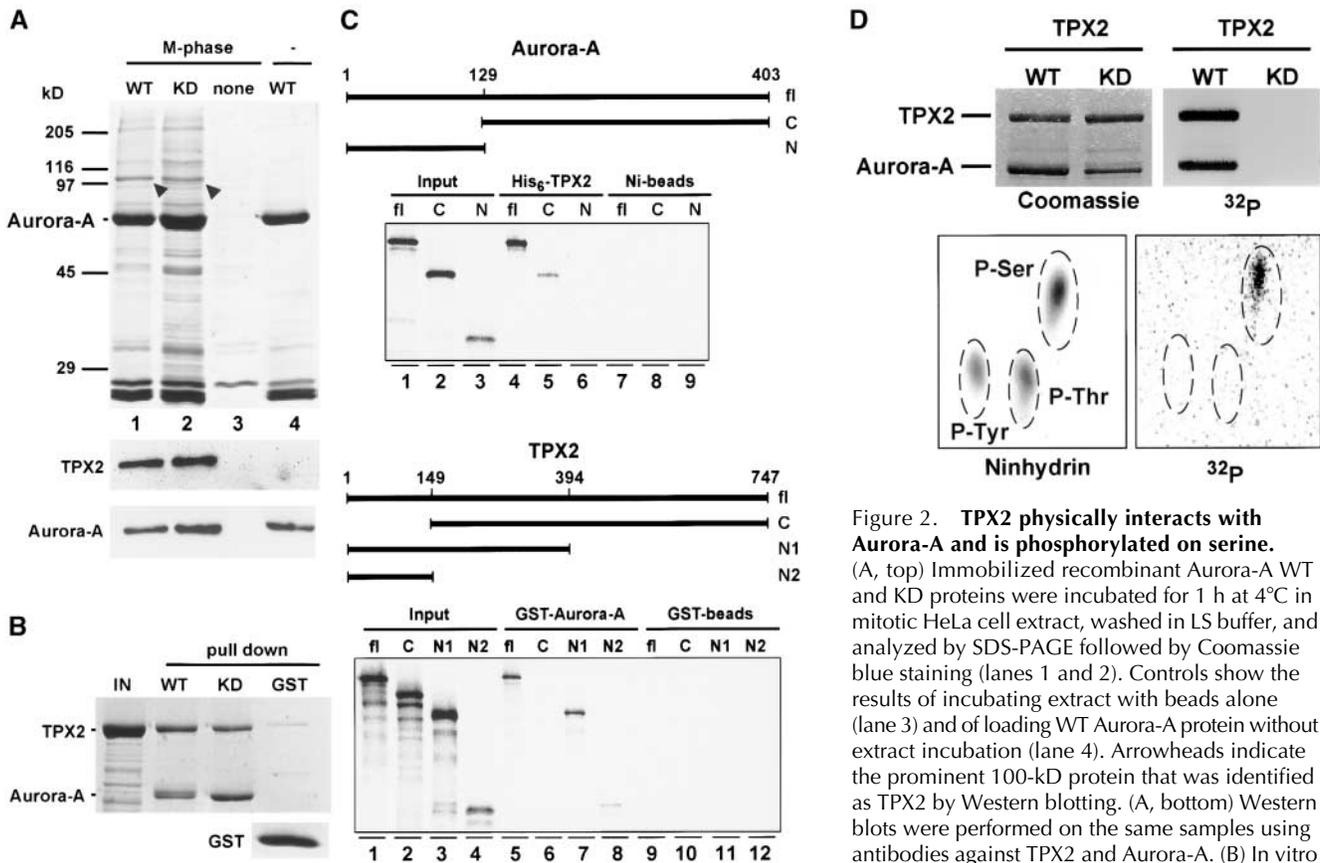


Figure 2. TPX2 physically interacts with Aurora-A and is phosphorylated on serine.

(A, top) Immobilized recombinant Aurora-A WT and KD proteins were incubated for 1 h at 4°C in mitotic HeLa cell extract, washed in LS buffer, and analyzed by SDS-PAGE followed by Coomassie blue staining (lanes 1 and 2). Controls show the results of incubating extract with beads alone (lane 3) and of loading WT Aurora-A protein without extract incubation (lane 4). Arrowheads indicate the prominent 100-kD protein that was identified as TPX2 by Western blotting. (A, bottom) Western blots were performed on the same samples using antibodies against TPX2 and Aurora-A. (B) In vitro binding of recombinant TPX2 (IN) to immobilized

GST-tagged Aurora-A WT and KD proteins, or GST for control. (C) Mapping the interaction domains on Aurora-A and TPX2. Fragments of Aurora-A and TPX2, as indicated schematically, were produced by IVT, and binding to His₆-TPX2 (top) or GST-Aurora-A (bottom) was assayed. Protein complexes were recovered on Ni-Sepharose beads and glutathione beads, respectively; Ni-Sepharose beads or GST-coated glutathione beads were used for control. Autoradiographs show corresponding amounts of input samples and proteins recovered in protein complexes. (D) In vitro kinase assay was performed with recombinant GST-tagged Aurora-A and recombinant human TPX2 in the presence of [γ -³²P]ATP. Both the Coomassie blue-stained gel (left) and the autoradiograph (right) are shown. (D, bottom) Phosphoamino acid analysis of ³²P-labeled TPX2 (right); ninhydrin staining (left) shows the migration of phosphoamino acid standards.

and mass spectrometry (not depicted). No TPX2 was seen in control experiments using glutathione beads alone (Fig. 2 A, bottom, lanes 3) or in the Aurora-A input (Fig. 2 A, bottom, lanes 4). Most importantly, the extent of binding of TPX2 to WT or KD Aurora-A was virtually identical (Fig. 2 A, compare lanes 1 and 2). Thus, WT and KD Aurora-A have a similar affinity for TPX2.

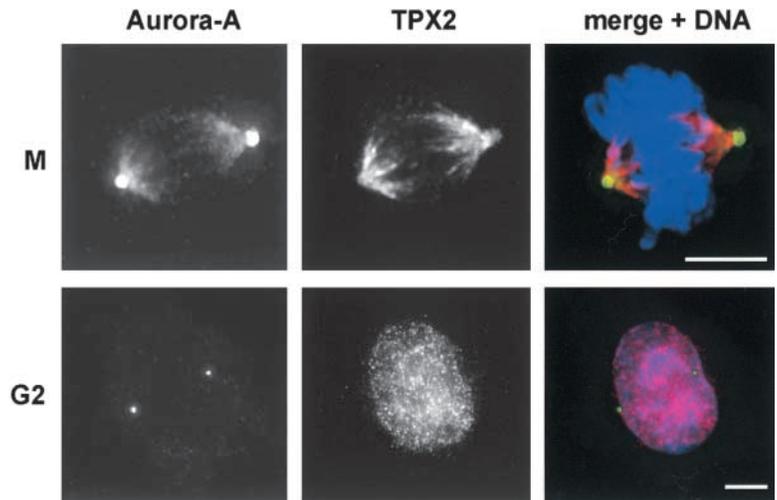
To establish whether the interaction between Aurora-A and TPX2 was direct or indirect, in vitro binding assays were performed using immobilized recombinant Aurora-A kinases and soluble, recombinant TPX2 tagged with the IgG binding domain of protein A. As shown in Fig. 2 B, TPX2 bound to WT or KD Aurora-A in near stoichiometric amounts, but virtually no TPX2 bound to GST-coated control beads. To map the interacting domains of the two proteins, binding studies were then performed with in vitro transcribed/translated fragments of Aurora-A and TPX2 (Fig. 2 C). The two full-length proteins bound to each other as expected, and no binding occurred to control beads. Binding to full-length TPX2 was also observed with the catalytic, COOH-terminal part of Aurora-A but not with the NH₂-terminal domain (Fig. 2 C, top). In a converse type of experiment (Fig. 2 C, bottom), the NH₂-terminal 394 amino acids of TPX2 (N1)

were able to bind full-length Aurora-A, and weak binding was also observed with the first 149 amino acids of TPX2 (N2). In contrast, no binding occurred with the COOH-terminal part (Fig. 2 C, C; residues 149–747). These data show that the catalytic domain of Aurora-A interacts directly with the NH₂-terminal domain of TPX2.

TPX2 is a substrate of Aurora-A

To investigate whether TPX2 is a substrate of Aurora-A, in vitro kinase assays were performed. Upon incubation of recombinant, protein A-tagged TPX2 with Aurora-A in the presence of [γ -³²P]ATP, TPX2 was readily phosphorylated by WT Aurora-A but not the KD mutant (Fig. 2 D). No incorporation of ³²P into the protein A tag was observed (not depicted). As determined by phosphoamino acid analysis, phosphorylation of TPX2 occurred on serine residues (Fig. 2 D). Human TPX2 has been reported previously to be phosphorylated in intact cells (Heidebrecht et al., 1997), and similarly, *Xenopus* TPX2 was shown to be phosphorylated specifically in M phase extracts (Wittmann et al., 2000). Although the kinase(s) responsible for these phosphorylations have not been identified previously, our present data identify Aurora-A as a likely regulator of TPX2. At present, we can only spec-

Figure 3. TPX2 and Aurora-A colocalize on the spindle. Indirect immunofluorescence micrographs of HeLa S3 cells stained for Aurora-A (left) and TPX2 (middle). A merge of the two images together with DAPI staining of DNA is shown in the right panel. The top row shows a representative metaphase cell; the bottom row shows a representative G2 phase cell. Bars, 10 μ m.



ulate on the physiological significance of TPX2 phosphorylation, but it is attractive to postulate that this modification could regulate the interaction of TPX2 with other spindle-associated proteins. In future studies, it will be important to map the phosphorylation sites within TPX2 and analyze the phosphorylation state of each site through the cell cycle.

TPX2 and Aurora-A partially colocalize in vivo

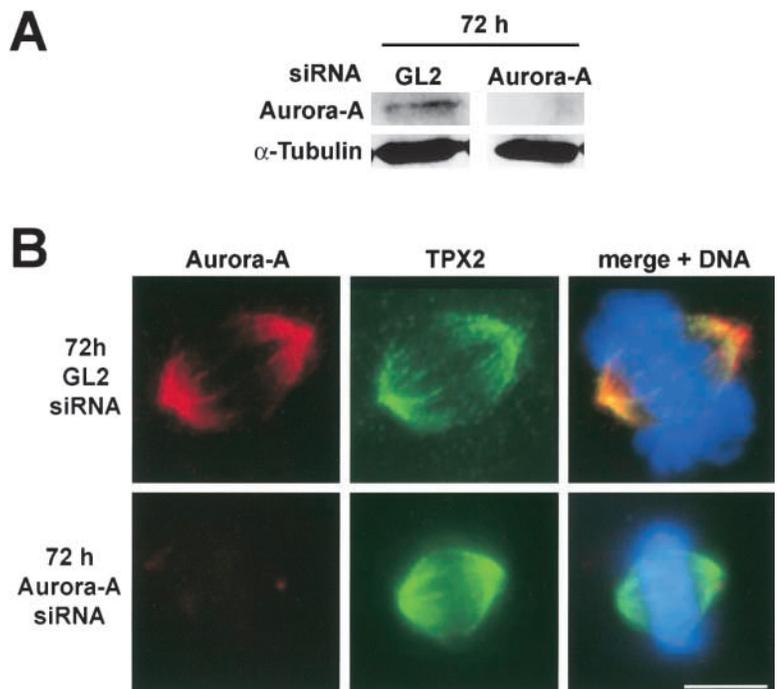
In previous studies, both TPX2 and Aurora-A have been localized independently to the spindle apparatus (Glover et al., 1995; Heidebrecht et al., 1997; Bischoff et al., 1998; Roghi et al., 1998; Schumacher et al., 1998; Wittmann et al., 2000). To compare the subcellular localization of these two proteins more directly, double indirect immunofluorescence microscopy was performed on HeLa and U2OS cells. In both cell types, a substantial colocalization of Aurora-A and TPX2 could be seen on the spindle apparatus. In contrast,

spindle poles were strongly positive for Aurora-A but only weakly stained by anti-TPX2 antibodies (Fig. 3, top row). Similarly, centrosomes in G2 cells were positive for Aurora-A but not for TPX2 (Fig. 3, bottom row). Thus, although both TPX2 and Aurora-A localize to spindle MTs, only Aurora-A is prominent at centrosomes and spindle poles.

TPX2 is required for recruiting Aurora-A to spindle MTs

To explore the functional significance of the interaction between Aurora-A and TPX2 in HeLa cells, the two corresponding genes were silenced by siRNA (Elbashir et al., 2001). A 72-h treatment with a duplex oligonucleotide specific for Aurora-A resulted in a substantial, albeit not complete, depletion of the kinase (Fig. 4, A and B). As a result, a significant increase in apoptotic events could be seen (not depicted). The remaining Aurora-A-depleted cells did not arrest in the cell cycle and bipolar spindles did form, al-

Figure 4. TPX2 localization is independent of Aurora-A. (A) Western blot analysis of total extracts from HeLa cells treated for 72 h with a siRNA duplex targeting Aurora-A or a control duplex (GL2). Filters were probed for Aurora-A and α -tubulin as a loading control. (B) HeLa S3 cells were treated for 72 h with an Aurora-A-specific siRNA duplex (top row) or a control duplex (GL2; bottom row) and then fixed and stained with antibodies against Aurora-A (left) and TPX2 (middle). A merge of the two images together with DAPI staining of DNA is shown on the right. Bar, 10 μ m.



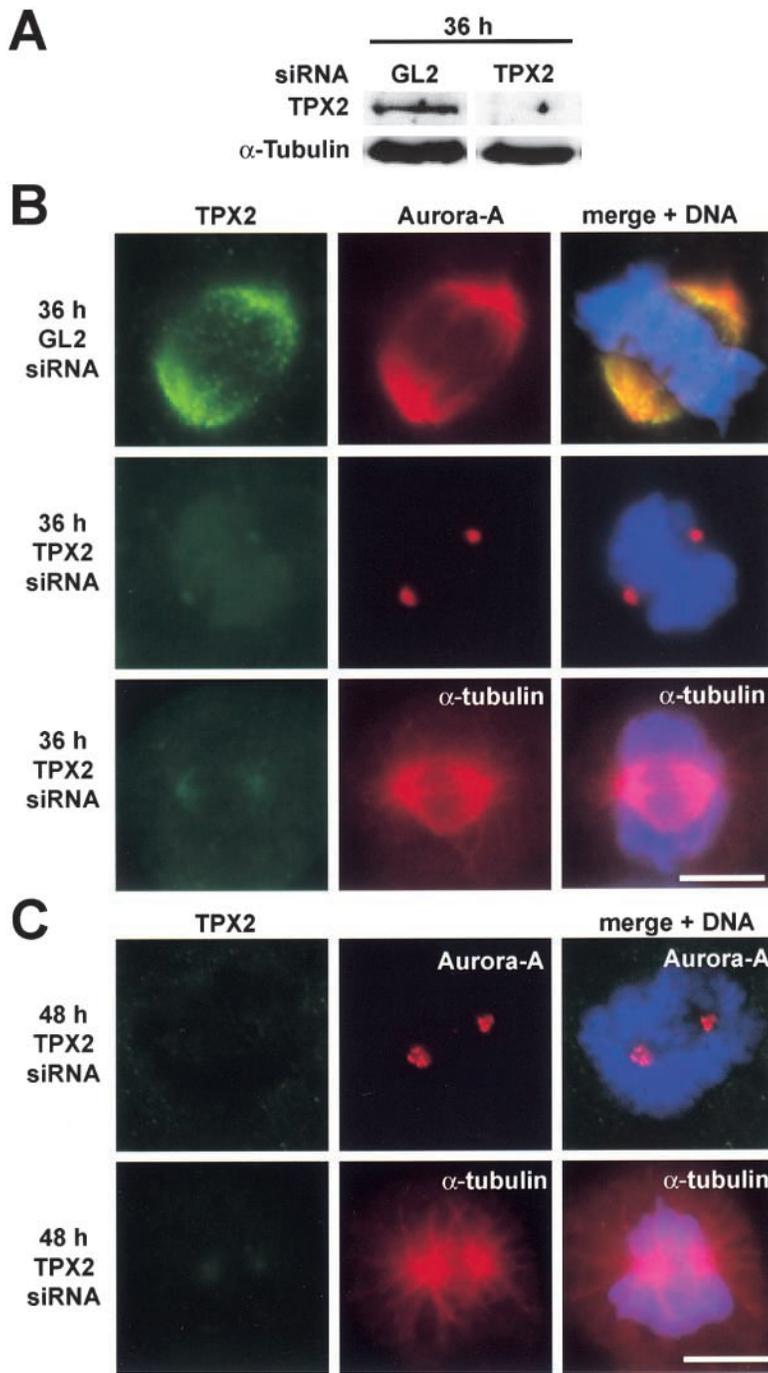


Figure 5. TPX2 localizes Aurora-A to the spindle. (A) Western blot analysis of total extracts from HeLa cells treated for 36 h with a TPX2-specific siRNA duplex or a control duplex (GL2). Filters were probed for Aurora-A and α -tubulin as a loading control. (B and C) HeLa S3 cells were treated for 36 (A) or 48 h (B) with TPX2-specific siRNA duplex or a control duplex (GL2) and then fixed and stained with the antibodies indicated. Right panels show merged images, including DNA staining by DAPI. Bars, 10 μ m.

though tubulin staining revealed that the poles were often broader than usual (not depicted). Of particular interest in the present context, depletion of the bulk of Aurora-A did not detectably interfere with the localization of TPX2 to spindle MTs (Fig. 4 B).

With the duplexes chosen here, the elimination of TPX2 by siRNA was more efficient than that of Aurora-A. The bulk of TPX2 had disappeared already after 36 h (Fig. 5, A and B), and by 48 h, the protein was almost undetectable (Fig. 5 C). Staining of MTs in TPX2-depleted cells showed that spindle formation was progressively impaired, and beginning at \sim 16 h after TPX2 siRNA treatment, many cells rounded up and arrested in a prometaphase-like stage (unpublished data). Most interestingly, the localization of Au-

rora-A was profoundly altered in TPX2-depleted cells. By 36 h of TPX2 depletion, Aurora-A was completely absent from MTs, although it could readily be detected at spindle poles (Fig. 5 B, middle row). This result could not be attributed to an absence of MTs, since these could readily be visualized by staining with anti- α -tubulin antibodies (Fig. 5 B, bottom row). By 48 h, spindle poles displayed increasing fragmentation, and again these fragmented poles stained positively for Aurora-A (Fig. 5 C). MT arrays were still present in these cells, but they were highly abnormal and, as observed already at the 36 h time point, devoid of Aurora-A (Fig. 5 C). Together, these results clearly indicate that TPX2 is specifically required for the association of Aurora-A with spindle MTs but not for its localization to spin-

dle poles. Conversely, the spindle association of TPX2 does not depend on the presence of Aurora-A.

In conclusion, we describe the identification of the spindle-associated MT-binding protein TPX2 as a novel interacting partner and candidate substrate of Aurora-A. In particular, we show that the association of Aurora-A with spindle MTs requires TPX2 but not vice versa. Also, Aurora-A is able to localize to spindle poles without TPX2. These findings indicate that TPX2 mediates specifically the dynamic interaction between Aurora-A and spindle MTs. Conversely, they raise the possibility that TPX2 function is regulated by Aurora-A kinase.

Materials and methods

Production of recombinant proteins and antibodies

Aurora-A WT and K162R cDNAs (Meraldi et al., 2002) were GST tagged and subcloned into pVL13GST93 (Amersham Biosciences). Recombinant baculoviruses were produced using the BaculoGold kit according to the manufacturer (BD Pharmingen). Sf9 cells were lysed in lysis buffer (LB; 10 mM Hepes, pH 7.7, 150 mM NaCl, 5 mM EGTA, 1% NP-40) containing phosphatase and protease inhibitors as described (Stucke et al., 2002). GST-Aurora-A was subsequently isolated using glutathione-Sepharose™ 4B beads (Amersham Biosciences) and washed with LB containing 300 mM NaCl and then with kinase buffer (20 mM Hepes, pH 7.4, 150 mM KCl, 5 mM MnCl₂, 5 mM NaF, 1 mM DTT).

A rabbit antibody against human Aurora-A was raised using a COOH-terminal peptide (CQNKESASKQS) of human Aurora-A coupled to keyhole limpet hemocyanin as antigen. Antibodies were immunopurified on the antigenic peptide covalently bound to activated thiopropyl Sepharose™ (Amersham Biosciences). For expression of recombinant human TPX2, an NH₂-terminal protein A binding domain (zz domain) was fused to the TPX2 cDNA followed by a histidine tag from the pQE 60 (QIAGEN) vector. TPX2 protein was expressed in *Escherichia coli* and purified as described before (Gruss et al., 2001). A rabbit antibody against TPX2 was raised using the recombinant full-length human TPX2 protein. Polyclonal sera were affinity purified on the antigen covalently linked to AffiGel 10 (Biometra).

Cell lysates, immunoprecipitations, and immunoblotting

Cell extracts from nocodazole (100 ng/ml) arrested mitotic HeLa S3 cells were prepared as described previously (Stucke et al., 2002). For immunoprecipitations, cell extracts were incubated for 1 h with Aurora-A antibody followed by a 3-h incubation with Affi-Prep® Protein A beads (Bio-Rad Laboratories). Beads were subsequently washed three times in LS buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% NP-40) and boiled in SDS-PAGE sample buffer. As a negative control, the antibody was incubated for 30 min with a 100× molar excess of the antigenic peptide before the incubation. To precipitate TPX2 complexes, the monoclonal antibody mAb Ki-S2 (Heidebrecht et al., 1997) was bound to Protein G Sepharose™ 4 fast flow (Amersham Biosciences), incubated for 3 h in the cell extracts, washed in LS buffer, and boiled in gel sample buffer. For Western blotting, Ki-S2 (1:5) or the purified Aurora-A antibody (32 µg/ml) were used and detected by ECL SuperSignal® (Pierce Chemical Co.).

siRNA

For siRNA, the following target sequences were used: Aurora-A, ₇₂₅AUG CCC UGU CUU ACU GUC A₇₄₃; TPX2, ₁₄₄GAA UGG AAC UGG AGG GCU₁₆₁. Oligonucleotides (Dharmacon Research, Inc.) were annealed and transfected using oligofectamine (Life Technologies) as described (Elbashir et al., 2001). As a control, the GL-2 duplex, targeting the luciferase gene, was used (Elbashir et al., 2001).

Immunofluorescence microscopy

Immunofluorescence microscopy was performed on HeLa S3 cells grown on coverslips fixed for 6 min in -20°C methanol. Sequential incubation of primary and secondary antibodies was done in PBS containing 3% BSA. Immunofluorescence microscopy, image acquisition, and processing was performed as described previously (Stucke et al., 2002). Antibodies used are as follows: purified rabbit α-Aurora-A (32 µg/ml), Ki-S2 (1:2) (Heidebrecht et al., 1997), rabbit α-TPX2 (20 µg/ml), mouse mAb α-tubulin (1:3,000) (Sigma-Aldrich), Texas red-conjugated goat α-mouse IgG (1:1,000) (Sigma-

Aldrich), and Alexa488-conjugated goat α-rabbit IgG (1:1,000) (Molecular Probes). DNA was stained with DAPI (Sigma-Aldrich) (2 µg/ml).

In vitro protein binding and kinase assays

In vitro binding assays were performed by incubating recombinant GST-tagged Aurora-A bound to glutathione-Sepharose with soluble recombinant TPX2 for 1 h at 30°C in kinase buffer containing 10 µM ATP. Subsequently, the beads were washed three times in LS buffer, and proteins were analyzed by SDS-PAGE followed by Coomassie blue staining. For in vitro kinase assays, recombinant Aurora-A WT and KD bound to glutathione-Sepharose were incubated with recombinant TPX2 in kinase buffer including 0.1 µCi [³²P]ATP and 10 µM ATP for 30 min at 30°C before reactions were stopped by the addition of SDS-PAGE sample buffer. Autoradiographs were made using a PhosphorImager (Fuji-Film).

Phosphoamino acid analysis of in vitro-phosphorylated TPX2 protein was performed by acid hydrolysis and thin layer chromatography as described (Boyle et al., 1991). Fragments of TPX2 and Aurora-A gene were generated by PCR and subcloned into the pcDNA3.1(-)myc3 (Invitrogen) vector. In vitro transcription/translation (IVT) was performed in the presence of ³⁵S methionine using TnT®-coupled reticulocyte lysate (Promega) and T7 RNA polymerase (Promega). IVT products were diluted three times in PBS and incubated for 3 h at 4°C with an excess of recombinant TPX2 bound to Ni-Sepharose (QIAGEN) or GST-Aurora-A bound to glutathione beads. Beads were washed four times with LS buffer and analyzed by SDS-PAGE followed by autoradiography.

Mass spectrometry

Proteins were in-gel-digested with sequencing-grade porcine trypsin (Promega) essentially as described (Shevchenko et al., 1996). Peptide mass fingerprinting was performed on the extracted peptides using a MALDI-TOF instrument (Reflex III; Bruker) and probability-based database searching (Perkins et al., 1999).

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