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A novel role for TPX2 as a scaffold and co-activator protein of the Chromosomal Passenger Complex

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Abstract

Aurora B kinase forms the enzymatic core of the Chromosomal Passenger Complex (CPC) and is a master regulator of mitosis. Understanding the regulation of Aurora B is critical to illuminate its role in mitosis. INCENP, Survivin and Borealin have all been known to promote Aurora B activation. In this study, we have identified the Aurora A activator protein TPX2 as a novel scaffold and co-activator protein of the CPC. Studies utilizing M-phase *Xenopus* egg extracts (XEE) revealed that the immunodepletion of endogenous TPX2 from XEE decreases Aurora B-Survivin and Aurora B-INCENP interactions, leading to a consequent reduction in Aurora B activity. Further, residues 138 to 328 of *Xenopus* TPX2 (TPX2 B) are sufficient to enhance Aurora B-Survivin association and Aurora B kinase activity *in vitro*. Importantly, experiments with pancreatic cancer cell lines suggest that this mechanism of Aurora B activation by TPX2 is likely to be conserved in human cells. Strikingly, the overexpression of human TPX2 B in HeLa cells causes defects in metaphase chromosome alignment and INCENP localization. Thus, in addition to its already established role as an Aurora A activator, our data support the role of TPX2 as a novel co-activator of Aurora kinase B.

Keywords

TPX2; Aurora B; Survivin; INCENP; mitosis; Chromosomal Passenger Complex

1. Introduction

Mitosis is a fundamental biological process that needs to be carried out accurately, and with minimum errors to ensure the generation of two genetically identical daughter cells. Any defect in this process can lead to genomic instability, which is a hallmark of cancer cells [1]. Aurora kinases are master regulators of mitosis [2–4] and are promising targets for anti-cancer therapy (Reviewed by [5]).

Humans possess three Aurora kinases – A, B and C. Aurora A kinase plays a role in centrosome separation and maturation, and in ensuring that a proper bipolar spindle forms to separate mitotic chromosomes. Aurora B kinase, on the other hand, is the enzymatic component of a complex called the Chromosomal Passenger Complex (CPC). The CPC consists of three other proteins besides Aurora B, these include: Inner Centromere Protein (INCENP), Survivin and Borealin. The activity of Aurora B as the catalytic core of the CPC is necessary for accurate chromosome segregation (by mediating proper kinetochore-microtubule (MT) attachments), chromosome condensation and congression, cytokinesis,

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and chromosome-associated spindle assembly (Reviewed by [6–9]). Aurora C kinase is the least studied of all the three Aurora kinases as its expression is mainly restricted to the germ cells [10–14]. Aurora C is known to compensate for the loss of Aurora B activity [15,16].

Targeting protein for *Xenopus* kinesin-like protein 2 (TPX2) is a well known Aurora A activator protein [17,18]. TPX2 is also necessary for targeting Aurora A to the spindle MTs [19]. The activity of TPX2 is required for proper spindle assembly during mitosis [20]. TPX2 binds to the catalytic kinase domain of Aurora A. This binding of TPX2 triggers a conformational change in Aurora A that promotes Aurora A auto-phosphorylation on threonine (Thr) 288 (human) or Thr 295 (*Xenopus*) and its subsequent activation. Additionally, the binding of TPX2 to Aurora A also prevents the association of the phosphatase PP1 with Aurora A, further enhancing Aurora A activation [17,18,21].

Many proteins, including INCENP, Borealin, Tausled-like kinase 1 (TLK1), Chk1, telophase-disc 60 (TD-60), MTs, and End-binding protein 1 (EB1), activate Aurora B [22–30]. The binding of Aurora B to INCENP increases the kinase activity of Aurora B by promoting its autophosphorylation on Thr 232 (human) or Thr 248 (*Xenopus*). Upon activation by INCENP, Aurora B binds the C-terminal IN-box of INCENP and phosphorylates the Thr-Ser-Ser (TSS) motif within the IN-box. This phosphorylation of INCENP on TSS residues further enhances the kinase activity of Aurora B [23,24,31,32]. Importantly, several studies have implicated Survivin as an Aurora B activator in both *Xenopus* egg extracts (XEE) and human cells [33–37]. A recent paper by Chu and colleagues clearly demonstrates that the phosphorylation of Survivin on Ser 20 by Polo-like kinase 1 enhances Aurora B activity [36]. Thus, Survivin is now a well-established Aurora B activator.

Studies by Murata-Hori et. al, revealed that GFP-Aurora B localizes to the spindle poles – a region where TPX2 is also housed [38]. In another study by Tseng and colleagues, endogenous Aurora B staining was detected at the spindle poles during metaphase. Further, the specificity of this spindle pole localization of Aurora B was confirmed by its knockdown using RNA interference [39]. These observations indicate that TPX2 and Aurora B likely co-localize at the spindle poles during metaphase. Additionally, during metaphase, TPX2 also localizes to the kinetochore MTs, a region where Aurora B is present [40,41]. Moreover, both TPX2 and Aurora B localize at the midbody during telophase [20,22]. These observations point towards existence of a signaling cross-talk between the Aurora A activator protein TPX2 and Aurora B during one or more stages of mitosis. Since both Aurora B and TPX2 proteins are indispensable for mitosis, it is critical to study whether they can communicate with each other to bring about proper mitosis. Indeed, our study reports a novel role for TPX2 as an Aurora B co-activator by serving as a scaffold for assembly of the CPC.

2. Materials and Methods

Preparation of CSF-arrested XEE

CSF-arrested M-phase XEE were prepared using the protocol from (Tsai et. al, 2003) [17].

Cell culture

HeLa, 293T, Panc-1 and CFPAC-1 cells were maintained by culturing them in Dubelcco's Modification of Eagle's Medium (DMEM) (Gibco-Invitrogen, NY, USA) supplemented with 10% Fetal Bovine Serum (HyClone, Utah, USA) at 37°C and 5% CO₂. HeLa and 293T cells were obtained from Dr. Manabu Furukawa. Panc-1 and CFPAC-1 cells were a kind gift from Dr. Pankaj Singh.

Transfections and synchronizations

For IPs in HeLa cells, HeLa cells were first transfected with myc, myc-FL TPX2 or myc-TPX2 B using Lipofectamine 2000 (Invitrogen, New York, USA) according to the manufacturer's protocol for 12 hours. After 12 hours, the medium containing the Lipofectamine reagent was replaced by new medium containing 100 ng/ml nocodazole (Sigma-Aldrich, Missouri, USA) for 12 hours to synchronize the cells in early mitosis and then the cells were harvested for lysis. 293T, Panc-1 and CFPAC-1 cells were also synchronized with 100 ng/ml nocodazole for between 12 to 14 hours. For the immunofluorescence experiments, HeLa cells were transfected using Lipofectamine 2000 (Invitrogen, New York, USA) according to the manufacturer's protocol for 18 hours.

Constructs

Kindly refer to the supplementary materials and methods section.

Xenopus Aurora B IPs

CSF-arrested M-phase *Xenopus* egg extracts (XEE) were used to perform Aurora B IPs. 100 μ l of XEE was used for each IP. The IPs were performed in the presence of 30 μ g of the respective protein {GST (control), GST-FL TPX2, GST-TPX2 A, GST-TPX2 B, GST-TPX2 C or GST-TPX2 D} per 100 μ l of XEE. For the IPs, Dynabeads Protein A (Invitrogen, NY, USA) beads were first crosslinked to an in-house *Xenopus* Aurora B antibody using the crosslinker BS3 (Thermo Fisher Scientific, Illinois, USA) according to the manufacturer's protocol. The antibody cross-linked beads were then washed and incubated with egg extract containing the above-mentioned proteins. The IPs were performed at room temperature (to enable microtubule polymerization to occur) for 1 hour. The beads were then washed with 1X XB buffer, re-suspended in SDS sample buffer and analyzed by western blotting. The same protocol was followed for performing Aurora B IPs in either mock-depleted or TPX2-depleted XEE. However, in this case, 80 μ l of XEE was used for each IP.

GST IPs in XEE

For GST IPs, 2 μ g of either GST or GST-FL TPX2 was added to 100 μ l of XEE. These egg extracts were incubated with GST antibody-coated Dynabeads protein G (Invitrogen, NY, USA) for 1 hour at 4°C. The immunoprecipitates were washed with 1X XB buffer, resuspended in 2X SDS sample buffer and denatured by heating at 100°C for 2 minutes.

Immunoprecipitations (IPs) in mammalian cells

IPs were performed in nocodazole-synchronized HeLa, CFPAC-1 and Panc-1 cells. All the cells were harvested and lysed using NP-40 lysis buffer. The protein concentration was measured using the Bio-Rad protein assay kit (Bio-Rad Laboratories, California, USA) and 1 mg of cell lysate was used to perform each IP. For the Myc IPs, Dynabeads Protein G (Invitrogen, NY, USA) were first conjugated to the Myc antibody (Ab) (a kind gift from Dr. Keith Johnson) for at least 1 hour at 4°C. The beads were then washed with NP-40 lysis buffer and incubated with the respective cell lysates at 4°C for 1 hour. For Aurora B IPs in pancreatic cancer and 293T cell lines, the lysates were first incubated with Aurora B Ab (Abcam, Massachusetts, USA) overnight at 4°C. Following this, washed Dynabeads Protein A (Invitrogen, NY, USA) were added to the lysates and incubated for approximately 2 hours. Following the immunoprecipitation, all IP samples were washed with NP-40 lysis buffer, re-suspended in SDS sample buffer and analyzed by western blotting.

Protein expression

To generate recombinant proteins, constructs were bacterially expressed in Rosetta competent E-coli cells. For protein expression, an overnight 10 ml culture grown in Terrific Broth (TB) + 1% glucose was first pelleted. The supernatant was discarded and the pellet was re-suspended in 1 liter of TB and placed in a shaker for 2 hours at 37°C. Following this step, the culture was incubated at 23°C (room temperature) for 1 hour with shaking. After this, protein expression was induced by addition of 0.4 mM IPTG for 4 to 5 hours.

Protein purification

All proteins were purified using affinity chromatography. For purification, the bacterial pellets were lysed using Lysis Buffer (2X PBS + 10% glycerol + 0.5% Triton X-100 + 1 mM DTT + 1 mM PMSF). Additionally, either 10 mM EDTA or 10 mM Imidazole was included in the lysis and wash buffers for GST-tagged and His-tagged proteins respectively. Following lysis, the proteins were bound to the respective resins and the resins were washed with Wash Buffer (2X PBS + 10% glycerol + 1 mM DTT). The proteins were then eluted from the resin by addition of the Elution buffer (15 mM Glutathione in Wash Buffer, pH= 8.5 for GST-tagged proteins or 200 mM Imidazole in Wash Buffer, pH= 7.4 for His-tagged proteins). Upon elution, the proteins were desalted using Sephadex G-25 M columns (GE Healthcare, New Jersey, USA) and reconstituted in desalting buffer (1X Xenopus Buffer + 10% Glycerol + 1mM DTT). The proteins were further concentrated using Vivaspin columns (ISC Bioexpress, Utah, USA) according to the manufacturer's protocol, aliquoted, flash-frozen in liquid nitrogen and stored at -80°C until use.

In vitro binding assays

For *in vitro* binding assays, recombinant, bacterially expressed and purified Survivin and Aurora B proteins were mixed in the presence of 0, 1, 5 or 30 µg of myc-TPX2 B protein. The samples were incubated on a rotator at 4°C for 1 hour to allow Aurora B-Survivin protein complexes to form. Following this, 20 µl of either IgG-conjugated or Aurora B antibody-conjugated Dynabeads Protein A (Invitrogen, NY, USA) were added to the samples and incubated on the rotator at 4°C for 1 hour. An excess of BSA was added to all samples to prevent non-specific binding of the proteins to the beads. Next, the beads were washed 3X with 1X XB buffer and resuspended in 2X SDS buffer for analysis.

In vitro kinase assays

5 µg of Histone H3 (HH3) (Roche Applied Science, Indiana, USA) was mixed with either kinase buffer (XB+20 mM MgCl₂+ 10 mM ATP+ 1 mM DTT) alone or with recombinant, bacterially expressed and purified CPC proteins (Aurora B, INCENP, Survivin and Borealin) in the presence or in the absence of myc-TPX2 B protein in kinase buffer. The total volume of each reaction was 20 µl. The reactions were incubated at room temperature for either 5 or 10 minutes. At each timepoint, the reactions were stopped by addition of 5 µl of 4X SDS Sample buffer followed by heating at 100°C for 3 minutes. The samples were then analyzed by western blotting.

TPX2 Immunodepletions and add backs

XEE were immunodepleted according to the protocol from (Wittmann et. al, 2000) [20] using an in-house *Xenopus* TPX2 antibody. Following immunodepletion, either buffer or ~1 µg of purified GST-FL TPX2 protein were added to 80 µl of the immunodepleted egg extract.

Densitometry

ImageJ (<http://rsb.info.nih.gov/ij/>, National Institutes of Health, Maryland, USA) was used to quantify all the western blot data [42].

Immunofluorescence

For Immunofluorescence analysis, HeLa cells were first seeded on glass coverslips. 18 hours after transfection with the appropriate constructs, the cells were fixed onto the coverslips using cold methanol (-20°C) for 10 minutes. Following this, the cells were washed with 1X PBS, permeabilized with 0.1% Triton-X 100 and washed again with 1X PBS. The coverslips were then blocked in 4% BSA for 1 hour. This was followed by incubation with the primary antibody for 2 to 3 hours, washing off of the excess primary antibody and incubation with the secondary antibody for 30 minutes. After incubation with the secondary antibody, the coverslips were washed thrice again and mounted on glass slides. For the co-localization studies in MCF7 cells, the permeabilization step was skipped from the above-mentioned protocol.

Microscopy and Imaging software

For imaging immunofluorescence slides, we utilized an upright, inverted, Axiovert 200M Zeiss fluorescence microscope (Carl Zeiss, New York, USA). The Slidebook 4.2 software (Intelligent Imaging Innovations, Colorado, USA) was used for analyzing and processing all immunofluorescence images.

Microtubule Pelleting Assays

100 microliters of CSF-arrested M-Phase *Xenopus* egg extracts were incubated with either buffer (control) or 0.3 $\mu\text{g}/\mu\text{l}$ protein of interest at room temperature to allow MTs to polymerize. The reaction was stopped by diluting the egg extract reaction in 8 ml of BRB-80+30% glycerol. This mixture was then overlaid on 5 ml of BRB-80+40% glycerol in a glass test-tube. Following this, the test-tube was centrifuged in a Beckman Coulter centrifuge (Beckman Coulter, Inc., California, USA) at 4750 rpm for 15 minutes. The MTs and MT-associated proteins formed a pellet at the bottom upon centrifugation. The supernatant was then aspirated off and the MT pellet was re-suspended in 2X SDS sample buffer and analyzed by western blotting.

Antibodies

Xenopus TPX2, Aurora B, INCENP and Survivin antibodies were raised in-house. Rabbit Anti-Human Aurora B and INCENP antibodies were purchased from Abcam (Abcam, Massachusetts, USA), antibodies against human Survivin and phospho-Histone H3 Ser10 were purchased from Cell Signaling (Cell Signaling Technology, Inc., Massachusetts, USA), anti-Histone H3 antibody was purchased from BioLegend (BioLegend, California, USA) and anti-Flag antibody was purchased from Sigma-Aldrich (Sigma-Aldrich, Missouri, USA). To monitor Aurora B activity by phosphorylation of Thr 232/248, we either used a rabbit anti-phospho-Aurora ABC antibody from Cell Signaling (Cell Signaling Technology, Inc., Massachusetts, USA) or an in-house mouse monoclonal antibody against phospho-Aurora A and B. The goat anti-GST antibody for GST-pulldowns was purchased from GE Healthcare (GE Healthcare, New Jersey, USA).

Constructs

The following constructs were made:

***Xenopus* GST-TPX2 (Full length)**-Full length TPX2 was cloned into pGEX 6P-2 vector using BamH1 and Not1 restriction sites (Tsai et. al, 2003). pcDNA3myc3 and pcDNA3-mCherry vectors were kind gifts from Dr. Manabu Furukawa.

Construct Name	Primers
<i>Xenopus</i> GST-TPX2 B (aa 138–328)	Forward primer- 5'-ATATGGATCCATCAAGAGCCAATCCACAAGC-3' Reverse primer- 5'-ATATCTCGAGTCACATCTTCACTGGGGAGGG-3'
<i>Xenopus</i> GST-TPX2 C (aa 320–478)	Forward primer- 5'-ATATGGATCCATGGAGGGACCCTCCCCAGTG-3' Reverse primer- 5'-ATATCTCGAGTCAAAGTGGCACCATCTCTTC-3'
<i>Xenopus</i> GST-TPX2D (aa 476–715)	Forward primer- 5'-ATATGGATCCATGGTGCCAGTTATCAAAGCC-3' Reverse primer (pGEX Reverse)- 5'-GGAGCTGCATGTGTCAGAGG-3'
Human myc-TPX2 (Full Length)	Forward primer- 5'-ATATGGATCCTGGGAGGAGGATCATCACAAGTAAAAGCTCTTATTCC-3' Reverse primer- 5'-ATATAAGCTTTTAGCAGTGGAATCGAGTGGA-3'
Human myc-TPX2 B (aa 173–359)	Forward primer- 5'-ATATGGATCCTGGGAGGAGGATCATCACAAGTAAAAGCTCTTATTCC-3' Reverse primer-5'-ATATCTCGAGTTAAGCACTGCCTTCTTCTCTGGC-3'
Human mCherry-TPX2 (Full Length)	Forward primer-5'-ATATGGATCCTGGGAGGAGGATCATCACAAGTAAAAGCTCTTATTCC-3' Reverse primer- 5'-ATATAAGCTTTTAGCAGTGGAATCGAGTGGA-3'
Human mCherry-TPX2B (aa 173–359)	Forward primer- 5'- ATATGGATCCTGGGAGGAGGATCAGGCAGTGCTCATCAAGATAC-3' Reverse primer- 5'-ATATCTCGAGTTAAGAAGATTGGAGGGTAACAGGTT-3'
<i>Xenopus</i> -His-INCENP (full length)	Forward primer- 5'-ATATGGATCCATGAACGATGCAGAGTGC-3' Reverse primer-5'-ATATAAGCTTTTCACTATTTGAGGCCATAACCCAC-3'
<i>Xenopus</i> -Borealin-Flag-His (full length)	PCR1: Forward primer- 5'-ATATCATATGATGCCGCCCAAGAGGAACAGAAAT-3' Reverse primer- 5'-TCATCCTTGTAATCGAGGGTATTCCCGTGG-3' PCR2: Forward primer- 5'-ATATCATATGATGCCGCCCAAGAGGAACAGAAAT-3' Reverse primer- 5'-ATATCTCGAGCTTATCGTCGTCATCCTTGTAATCG-3'
<i>Xenopus</i> -Aurora BHis (full length)	Forward primer- 5'-ATATCATATGATGTCTTACAAAGAGAATCTCAACCC-3' Reverse primer- 5'-ATATAAGCTTTTGTGATGGGTGGACTGGTAGA-3'
<i>Xenopus</i> -Survivin-His (full length)	Forward primer- 5'-ATATCATATGATGTACTCTGCCAAGAAGAGGTT-3' Reverse primer- 5'-ATATAAGCTTGTGGTCAAGATCTATGGAGCAAT-3'
<i>Xenopus</i> myc-TPX2 B (aa 138–328)	PCR1: Forward primer- 5'-TCATCTCAGAGGAAGATCTGATCAAGAGCCAATCCA-3' Reverse primer- 5'-ATATCTCGAGTCACATCTTCACTGGGGAGGG-3' PCR2: Forward primer- 5'-ATATCATATGGAGCAAAAGCTCATCTCAGAGGAAGATCTG-3'

Construct Name	Primers
	Reverse primer- 5'-ATATCTCGAGTCACATCTTCACTGGGGAGGG-3'

3. Results

3.1. TPX2 interacts with the CPC proteins Aurora B, INCENP and Survivin

To determine whether TPX2 can interact with Aurora B and other CPC proteins, we pulled down TPX2 from M-phase XEE by immunoprecipitation (IP). For this purpose, recombinant, bacterially expressed and purified GST (control) or GST-TPX2 proteins were added to Cytostatic factor (CSF) arrested XEE and GST IPs were performed. Both GST and GST-TPX2 could be successfully immunoprecipitated from XEE as shown in Fig 1A. GST-TPX2, being a high molecular weight protein is particularly susceptible to degradation during the purification process. The additional bands in the GST-TPX2 IP sample in Fig 1A represent the degradation products of GST-TPX2 protein. The CPC protein components Aurora B, INCENP and Survivin specifically associated with GST-TPX2 in M-phase XEE (Fig 1B). A blot for Aurora A, a well known TPX2 interacting protein was used as a positive control for the GST-TPX2 IP. Thus, the CPC complex proteins were identified as novel and specific interacting partners of TPX2.

3.2. Under endogenous conditions, TPX2 functions as an Aurora B activator

To determine the biological significance of the interaction between TPX2 and the CPC proteins, we immunodepleted endogenous TPX2 from XEE. Following this, either an IgG-depleted egg extract or a TPX2-depleted egg extract was used to perform Aurora B warm IPs (IPs were performed at room temperature to facilitate MT polymerization because MTs are likely to be required for complete Aurora B activation) (Fig 2) [30]. Thr 232/248 (human/*Xenopus*) phosphorylation of Aurora B correlates with the kinase activity of Aurora B [23,24,31,32]. It was noted that the depletion of TPX2 from XEE decreased Aurora B phosphorylation on Thr 248 indicating that, the reduction of the cellular level of TPX2 decreases Aurora B activity (Fig 2A,B). Further, the depletion of TPX2 also reduced the association of Aurora B with both its activators, Survivin and INCENP, although the decrease in association of Aurora B with Survivin was a more consistent effect (FIGS. 2A,C,D,E). More importantly, when recombinant GST-TPX2 was added back to the TPX2-depleted egg extract, it could completely rescue the decrease in Aurora B activity and the reduced interaction of Aurora B with Survivin and INCENP mediated by the loss of TPX2 from the egg extract (Fig 2C). This result suggests that, under endogenous conditions, TPX2 recruits the Aurora B activator proteins Survivin and INCENP to Aurora B, and increases Aurora B activity. Thus, TPX2 was found to serve as a co-activator for Aurora B under endogenous conditions.

3.3. Addition of an excess of FL TPX2 and TPX2 B also decreases the amount of active Aurora B and the association of Survivin with Aurora B

Since TPX2 was found to function as an Aurora B activator under endogenous conditions, we questioned whether the addition of an excess of TPX2 could further enhance Aurora B activation by TPX2. To test this, the effect of addition of 30 ug of full length (FL) *Xenopus* (x) TPX2 (greater than 20 times the level of endogenous TPX2) or its truncations (A, B, C and D) on Aurora B activity was monitored by performing Aurora B IPs as described earlier. TPX2 A was comprised of amino acids 1 to 140, TPX2 B of residues 138 to 328, TPX2 C of amino acids 320 to 478 and TPX2 D contained the C-terminal 476 to 715 residues of xTPX2

(Fig 3A). A GST blot to detect the association of these proteins with Aurora B showed that both FL TPX2 and TPX2 B could interact strongly with Aurora B (Fig 3B). Surprisingly, instead of increasing Aurora B activity, the addition of both of these proteins also decreased Aurora B phosphorylation at Thr 248 in Aurora B IPs, thereby denoting a reduction in Aurora B activity (Fig 3C,D). Thus, the addition of an excess of GST-FL TPX2 or GST-TPX2 B also reduced Aurora B activity. Further, the addition of an excess of TPX2 B to XEE was also sufficient to decrease the association of the Aurora B with both its activators, Survivin and INCENP (Fig 3E,F). Thus, either too little TPX2 (immunodepletion) or too much TPX2 (addition of an excess) alters Aurora B activity by causing the dissociation of Survivin and INCENP from Aurora B. This behaviour is typical of scaffold proteins. Therefore next, we questioned whether TPX2 acts as a scaffold protein for assembly of the CPC.

3.4. TPX2 B increases the association of Survivin with Aurora B *in vitro*

In the TPX2 immunodepletion assays that were performed in the XEE we determined that, the change in association of Aurora B with Survivin upon TPX2 depletion was more drastic than the change in its interaction with INCENP (Fig 2D,E). Therefore, we hypothesized that the phenotype of Aurora B inactivation upon TPX2 depletion occurs predominantly due to a decrease in Survivin-Aurora B interaction. To further test this hypothesis and to support the data obtained in XEE, we performed *in vitro* binding assays to monitor the Survivin-Aurora B interaction both in the presence and in the absence of TPX2.

TPX2 B mirrored the phenotype of altering Aurora B activity that was displayed by FL TPX2. Hence, we performed *in vitro* binding assays utilizing purified Aurora B, Survivin and TPX2 B proteins to investigate whether there was a change in Survivin-Aurora B interaction in the presence of TPX2 B. In these experiments, purified *Xenopus* His-Aurora B and Survivin-His proteins were mixed in the presence of 0, 1, 5 or 30 µg of myc-TPX2 B and the change in association of Survivin with Aurora B was examined by performing Aurora B IPs and blotting for Survivin (Fig 4A). Negative controls for the experiment were included to ensure that TPX2 B does not cause non-specific association of Survivin with Aurora B antibody (Ab) coated beads. We found that the addition of either 5 µg or 30 µg of TPX2 B increased the association of Survivin with Aurora B *in vitro* (Fig 4A). This provides further evidence for the existence of a model in which TPX2 acts as a scaffold to recruit Survivin to Aurora B.

3.5. TPX2 B increases Aurora B kinase activity *in vitro*

Upon observing an increased interaction of Survivin with Aurora B in the presence of TPX2 B, we wanted to determine whether this increased interaction translates to an increase in Aurora B kinase activity. To address this issue, Aurora B kinase assays were performed *in vitro*. The phosphorylation of Histone H3 Serine 10 (HH3 Ser 10), a well-known Aurora B substrate, was monitored as a marker of Aurora B kinase activity in these assays [43–47]. For these assays, either Histone-H3 (HH3) alone, HH3+CPC or HH3+CPC+TPX2 B were incubated at room temperature for either 5 or 10 minutes in kinase buffer. The reaction was stopped by adding SDS sample buffer and heating. We determined that at both 5 and 10 minutes, the addition of TPX2 B increased the phosphorylation of HH3 Ser 10, which is a marker of Aurora B kinase activity (Fig 4B). Thus, we concluded that the addition of TPX2 B increases Aurora B kinase activity *in vitro*.

3.6. The TPX2-CPC interaction is conserved in human cells

To determine whether the interaction between TPX2 and the CPC proteins is conserved in humans, IgG (control) or Aurora B IPs were performed in nocodazole-synchronized 293T cells. Aurora B interacted with TPX2 under endogenous conditions in these cells (Fig 5A).

A blot for INCENP served as a positive control for the Aurora B IP. The fact that TPX2 associates with Aurora B under endogenous conditions further highlights that, the observed interaction between TPX2 and Aurora B is not an effect of over-expression of TPX2. The association of TPX2 with Aurora B was further confirmed by performing myc IPs in nocodazole-synchronized mitotic HeLa cells that were transfected with either myc empty vector (control) or human myc-FL TPX2 (Fig 5B). The interaction between myc-FL TPX2 and the CPC proteins was maintained in HeLa cells (Fig 5B). Further, to evaluate whether the human equivalent of *Xenopus* TPX2 B (human TPX2 amino acids 173–359) also possesses the ability to interact with the CPC proteins, myc IPs were performed in nocodazole-synchronized HeLa cells that had been transfected with either myc or myc-TPX2 B. Significantly, human TPX2 B could also interact with the CPC proteins Aurora B, INCENP and Survivin similar to *Xenopus* TPX2 B (Fig 5C). Thus, the interaction between TPX2 and the CPC proteins is conserved in humans. Additionally, we also performed immunofluorescence assays to examine whether the interaction between TPX2 and the CPC proteins is likely to be biologically relevant in humans. Significantly, TPX2 co-localized with all the CPC proteins-Aurora B, Survivin and INCENP, at the spindle poles and kinetochore MTs during prophase/prometaphase and metaphase and at the midbody during telophase (Fig 5D,E,F). Importantly, these findings agree with the preexisting literature which suggests that Aurora B localizes to the spindle poles, kinetochore MTs and midbody during mitosis [22,38–40]. Thus, the fact that TPX2 co-localizes with the CPC proteins Aurora B, INCENP and Survivin during prophase/prometaphase, metaphase as well as telophase indicates that the interaction between TPX2 and the CPC proteins is likely to be biologically significant in humans.

3.7. Over-expression of the human equivalent of *Xenopus* TPX2 B in HeLa cells causes mitotic abnormalities that are reminiscent of Aurora B inactivation

To study whether similar to *Xenopus* TPX2 B, an over-expression of the human equivalent of *Xenopus* TPX2 B (aa 173–353) also produces phenotypes that are indicative of Aurora B inactivation, we transfected HeLa cells with human TPX2 B (Fig 6). Importantly, like *Xenopus* TPX2 B, this region of TPX2 lacks the Aurora A-interaction domain of TPX2. Therefore, the phenotypes that arise upon its over-expression likely represent an Aurora A-interaction independent effect of TPX2 over-expression. Remarkably, we noted that, an over-expression of mCherry-TPX2 B caused abnormal chromosome alignment (Fig 6A,B) and abnormal localization of INCENP during metaphase (Fig 6C,D). Approximately half of the cells that displayed an over-expression of human TPX2 B had abnormally aligned metaphase chromosomes (Fig 6B). Chromosome misalignment is a phenotype that is frequently observed upon the inhibition of Aurora B activity [48]. Therefore, it is likely that similar to *Xenopus* TPX2 B, an excess of human TPX2 B also causes the inactivation of Aurora B. Further, about 45% of the cells that were transfected with human TPX2 B also showed defects in INCENP localization during metaphase (Fig 6D). Strikingly, an over-expression of human TPX2 B led to a massive accumulation of INCENP at the spindle poles and yielded diffused INCENP staining on the DNA in metaphase, indicating its mislocalization during metaphase (Fig 6C, middle panel). Furthermore, extremely high levels of human TPX2 B caused an almost complete disappearance of INCENP from DNA (Fig 6C, bottom panel). These data indicate that, similar to the *Xenopus* system, an excess of human TPX2 B is also likely to dissociate INCENP from Aurora B (a behaviour that is typical of scaffold proteins), thereby, decreasing its activity. Therefore, TPX2 also likely serves as a scaffold protein for the CPC in humans.

3.8. Interaction with TPX2 correlates with increased Aurora B activity in pancreatic cancer cells

To determine whether TPX2 also promotes assembly and activation of the CPC in human cells, as was observed in the *Xenopus* system, Aurora B IPs were performed using human pancreatic cancer cell lines. Panc-1 and CFPAC-1 cell lines were chosen for performing Aurora B IPs because they differ in their expression levels of TPX2 (Fig 7A). CFPAC-1 expresses more than twice the cellular level of TPX2 than Panc-1. Upon performing Aurora B IPs with these cell lines, we determined that greater amounts of TPX2, Survivin and INCENP associated with Aurora B in the CFPAC-1 cell line as compared with the Panc-1 cell line, in spite of Survivin and INCENP being present at lower levels in the former (Fig 7B). Further, a CFPAC-1 Aurora B IP yielded over 2.3 fold more Thr 232-phosphorylated (active) Aurora B than a Panc-1 Aurora B IP. These results suggest that, similar to the *Xenopus* system, TPX2 also enhances the association of Survivin with Aurora B and increases Aurora B activity in human cells.

3.9. A model describing the mechanism of Aurora B activation by TPX2

Based upon the data that we have obtained thus far, we propose a model whereby, under endogenous conditions, TPX2 functions as a scaffold for assembly of the CPC complex and promotes Aurora B activation (Fig 8). When TPX2 is absent, Survivin and INCENP associate very weakly with Aurora B thereby causing a net decrease in Aurora B activity (Fig 2). At endogenous levels, TPX2 enhances the interaction of Survivin and INCENP with Aurora B, and therefore causes an increase in Aurora B activity. However, at very high cellular levels, TPX2 forms separate complexes with members of the CPC and sequesters them. This ultimately results in disruption of the CPC and a net decrease in Aurora B activity (Fig 3).

4. Discussion

We have uncovered a novel role for TPX2 as an Aurora B co-activator. TPX2 enhances Aurora B kinase activity by serving as a scaffold for assembly of the CPC complex (Fig 8). Under endogenous conditions, TPX2 facilitates the interaction of Aurora B with its activators-Survivin and INCENP, thereby increasing Aurora B activity (Fig 2).

A consistent interaction was detected between TPX2 and the CPC proteins Aurora B, INCENP and Survivin in both M-phase XEE as well as in three different human cell lines (293T, HeLa, and CFPAC-1) (FIGS. 1B, 2A, 2C, 5A, 5B, 5C, 7B). This suggests that the TPX2-CPC association is not cell-type specific and occurs in *Xenopus* as well as both human immortalized (293T) and cancer (HeLa and CFPAC-1) cell lines (FIGS. 1B, 2A, 2C, 5A, 5B, 5C, 7B). Importantly, the association of endogenous Aurora B, INCENP and Survivin with TPX2 was observed in 293T and CFPAC-1 cells as well as in XEE (FIGS. 5A, 7B, 2A, 2C). Furthermore, a weak interaction between endogenous TPX2 and Aurora B was also detected in the Panc-1 cells (Data not shown). Additionally, the depletion of endogenous TPX2 resulted in the disappearance of TPX2 from the endogenous Aurora B immunoprecipitate in XEE (Fig 2A,C). This confirms the specificity of the interaction between TPX2 and Aurora B.

Studies have already demonstrated that TPX2 and Aurora B localize to the same cellular compartments (spindle poles, kinetochore MTs and midbody) at the same time during mitosis [22,38–40]. In agreement with previous studies, in this study, we have also detected a co-localization of endogenous TPX2 with endogenous Aurora B, INCENP and Survivin proteins at the spindle poles and kinetochore MTs in early mitosis and at the midbody during telophase (Fig 5D,E,F). Significantly, we observed that the spindle pole localization of

INCENP is a very reproducible effect. We utilized two different INCENP antibodies against two different epitopes of human INCENP protein and observed the localization of endogenous INCENP at the spindle poles in both cases (J.I., unpublished data). Furthermore, this spindle pole localization of endogenous INCENP was seen in two different human cell lines (MCF7 and HeLa) as well as in *Xenopus* XLKW-G cells (Fig 5F and J.I., unpublished data). Moreover, we also observed that an over-expression of human TPX2 B caused a massive accumulation of INCENP at the spindle poles (Fig 6C). The mechanism by which TPX2 B causes this accumulation of INCENP at the spindle poles is still unclear and requires further probing. All these data indicate that the localization of INCENP to the spindle poles is specific and consistent.

Although we observed a distinct co-localization of Aurora B with TPX2 in MCF7 cells (Fig 5D), in concert with data from other groups, we have noted that, the localization of Aurora B to the spindle poles is often very weak. However, as mentioned earlier, a very consistent co-localization of TPX2 with all the CPC proteins was observed both at the kinetochore MTs as well as at the midbody (Fig 5D,E,F). All these data collectively suggest that the interaction of TPX2 with the CPC proteins is a specific and biologically significant association.

The conformation of TPX2 protein in the *Xenopus* system seems to be important for its interaction with the CPC proteins, since all domains of TPX2 interact with Aurora B to some extent in XEE (Fig 3B). Aurora A and Aurora B proteins share as much as 70% homology in their catalytic kinase domains [7]. The first forty-three amino acid residues of TPX2 are sufficient to interact with the kinase domain of Aurora A [21]. Importantly, we have determined that the region of TPX2 that does not interact with Aurora A (TPX2 B) is sufficient to interact with the CPC proteins in both *Xenopus* (Fig 3B) and human (Fig 5C) systems. This indicates that unlike its interaction with Aurora A, TPX2 does not likely interact with the kinase domain of Aurora B. Thus, the mode in which TPX2 interacts with Aurora B seems to be different from the manner in which it interacts with Aurora A. We predict that the association between TPX2 and Aurora B may be mediated indirectly via either Survivin or INCENP. Although, this prediction requires further experimental testing.

The addition of an excess of the Aurora A-interacting TPX2 A fragment did not cause the dissociation of Survivin from Aurora B. Only TPX2 B, that does not interact with Aurora A, could mediate the dissociation of Survivin from Aurora B (Fig 3H). This further suggests that the mechanism by which TPX2 activates Aurora B is different from the mode by which it activates Aurora A. Thus, our results indicate that TPX2 activates Aurora B indirectly by facilitating the association of Aurora B with its activator proteins, Survivin and INCENP.

Although we found that an excess of TPX2 B was sufficient to decrease Aurora B activity, other domains of TPX2 (TPX2 A and TPX2 D) also affected Aurora B activity (Fig 3G,I). However, addition of an excess of TPX2 D only reduced Aurora B activity in MT-pelleting assays but not in IPs (Fig 3I,G). Moreover, high levels of TPX2 A could inhibit Aurora B activity only in Aurora B IPs but not in MT-pelleting assays (Fig 3G,I). But most importantly, only TPX2 B caused a consistent and drastic alteration of Aurora B activity in both MT-pelleting assays and IP experiments (Fig 3G,I). Consequently, this protein was selected for our further assays.

We determined that the effect of TPX2 on the Aurora B-Survivin interaction was more consistent and pronounced than its effect on the Aurora B-INCENP interaction in XEE (Fig 2D,E). However, more importantly, the addition of an excess of the TPX2 B fragment of TPX2 could significantly and consistently reduce the Aurora B-INCENP interaction in XEE (Fig 3E,F). Additionally, our experiments with pancreatic cancer cell lines also demonstrate that the association of endogenous INCENP with Aurora B is much stronger in the

CFPAC-1 cell line which has higher endogenous levels of TPX2 and where more TPX2 is present in the Aurora B immunoprecipitate, than in the Panc-1 cell line. (Fig 7A,B). Taken together, all these results suggest that TPX2 facilitates an interaction between Aurora B and INCENP in both XEE and in human cell lines.

Borealin, another member of the CPC, is also known to contribute to Aurora B activation. Phosphorylation of Borealin by Mps1 increases Aurora B activity [25]. Unfortunately, due to lack of access to an antibody against *Xenopus* Borealin, we could not determine the effect of TPX2 on Aurora B-Borealin interaction. However, it will be interesting to study the effect of TPX2 on Borealin-Aurora B interaction in future. We speculate that TPX2 will also enhance the association of Borealin with Aurora B. The rationale for this speculation is that Borealin does not directly interact with Aurora B [49]. Borealin interacts with Aurora B indirectly via Survivin and INCENP [50]. Since TPX2 enhances the association of Survivin and INCENP with Aurora B, we would also expect a consequent increase in the interaction of Borealin with Aurora B in the presence of TPX2.

We have shown that the levels of TPX2 in human pancreatic cancer cell lines correlate strongly with how strongly Aurora B interacts with its activators – Survivin and INCENP in these cell lines (Fig 7). Moreover, we have also demonstrated that an over-expression of human TPX2 B gives rise to phenotypes that are indicative of inactivation of Aurora B (Fig 6). These data suggest that our scaffold mechanism is likely to be conserved in human cells. Further, although the endogenous levels of Aurora B, Survivin and INCENP are lower in the CFPAC-1 cell line as compared with the Panc-1 cell line (Fig 7A), more Survivin and INCENP associate with Aurora B in the CFPAC-1 cell line and consequently, Aurora B is more active in this cell line (Fig 7B). Thus, the expression level of TPX2, at least in this case, serves as a better predictive marker of Aurora B activity than the cellular levels of Aurora B, INCENP and Survivin. This observation is especially important because Aurora B inhibitors are currently being evaluated for their efficacy in cancer therapy. However, more cancer cell lines need to be tested to determine if this positive correlation between TPX2 levels and Aurora B activity is a common phenomenon in cancer cell lines. Based upon our data, we also propose that extremely high levels of TPX2 may be indicative of low Aurora B activity in human cell lines. However, this speculation requires further experimental evidence.

Recently, Aguirre-Portoles, et. al. generated TPX2 knockout and heterozygous mouse embryos [51]. In this study, the authors noted that TPX2-null MEFs exhibited defects in chromosome segregation and genome stability. Further, the TPX2-null cells also displayed an increase in binucleation and polyploidy, indicating that these TPX2-depleted cells failed to undergo cytokinesis. All these are phenotypes that are frequently observed upon the inactivation of Aurora B [48]. We have also found in our study that an over-expression of TPX2B causes abnormal chromosome alignment and the mislocalization of the Aurora B activator protein, INCENP during metaphase-phenotypes that denote decreased Aurora B activity (Fig 6). Therefore, the results from our work and the data from Aguirre-Portoles and colleagues provide evidence to support a possible model whereby, TPX2 serves as a scaffold protein to promote Aurora B activation to regulate chromosome alignment and cytokinesis. However, additional experiments are necessary to tease out exactly how the cross-talk between TPX2 and the CPC proteins regulates mitosis.

5. Conclusions

In conclusion, through our work, we have deduced the mechanism by which the Aurora A activator protein TPX2 facilitates the activation of Aurora B – a protein that is critical for proper chromosome alignment and cytokinesis. Specifically, studies in XEE have shown

that, under endogenous conditions, TPX2 increases the association of the Aurora B activator proteins: INCENP and Survivin with Aurora B, thereby increasing Aurora B activity. Moreover, either too much or too little TPX2 alters Aurora B activity. This behaviour is typical of scaffold proteins. Therefore, TPX2 functions as a scaffold protein for the assembly of the CPC. Additionally, this mechanism of Aurora B activation by TPX2 seems to be conserved in humans. Thus, in addition to its already well-established role as an activator of Aurora A kinase, TPX2 also mediates activation of Aurora kinase B. Hence, TPX2 functions as a dual activator of both Aurora kinases – A and B. Importantly, the signaling cross-talk between TPX2 and Aurora B seems to be required for the accurate completion of mitosis.

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Abbreviations

CPC	Chromosomal Passenger Complex
XEE	<i>Xenopus</i> egg extracts
INCENP	Inner Centromere Protein
TPX2	Targeting protein for <i>Xenopus</i> kinesin-like protein 2
MT/MTs	Microtubule/Microtubules
Thr	Threonine
Ser	Serine
IP	Immunoprecipitation
aa	Amino acid residues
AP	Alkaline phosphatase
HH3	Histone H3
Ab	Antibody
IF	Immunofluorescence

References

1. Lengauer C, Kinzler K, Vogelstein B. Nature. 1998; 396:643–649. [PubMed: 9872311]
2. Francisco L, Wang W, Chan C. Mol. Cell. Biol. 1994; 14:4731–4740. [PubMed: 8007975]
3. Glover DM. Cell. 1995; 81:95. [PubMed: 7720077]
4. Giet R, Prigent C. J. Cell. Sci. 1999; 112:3591–3601. [PubMed: 10523496]
5. Katayama H, Sen S. Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms. 2010; 1799:829–839.
6. Adams RR, Carmena M, Earnshaw WC. Trends Cell Biol. 2001; 11:49–54. [PubMed: 11166196]
7. Carmena M, Earnshaw WC. Nat. Rev. Mol. Cell Biol. 2003; 4:842–854. [PubMed: 14625535]
8. Carmena M, Ruchaud S, Earnshaw WC. Curr. Opin. Cell Biol. 2009; 21:796–805. [PubMed: 19836940]

9. Walczak CE, Cai S, Khodjakov A. *Nature Reviews - Molecular Cell Biology*. 2010; 11:91–102.
10. Kimmins S, Crosio C, Kotaja N, Hirayama J, Monaco L, Höög C, Van Duin M, Gossen J, Sassone-Corsi P. *Molecular Endocrinology (Baltimore)*. 2006; 21:726–739.
11. Dieterich K, Soto Rifo R, Faure A, Hennebicq S, Ben Amar B, Zahi M, Perrin J, Martinez D, Sèle B, Jouk P, Ohlmann T, Rousseaux S, Lunardi J, Ray P. *Nature Genetics*. 2007; 39:661–665. [PubMed: 17435757]
12. Tseng T, Chen S, Hsu Y, Tang T. *DNA Cell Biol*. 1998; 17:823–833. [PubMed: 9809744]
13. Kimura M, Matsuda Y, Yoshioka T, Okano Y. *Journal of Biological Chemistry*. 1999; 274:7334–7340. [PubMed: 10066797]
14. Hu H, Chuang C, Lee M, Tseng T, Tang T. *DNA and Cell Biology*. 2000; 19:679–688. [PubMed: 11098217]
15. Sasai K, Katayama H, Stenoien DL, Fujii S, Honda R, Kimura M, Okano Y, Tatsuka M, Suzuki F, Nigg EA, Earnshaw WC, Brinkley WR, Sen S. *Cell Motil. Cytoskeleton*. 2004; 59:249–263. [PubMed: 15499654]
16. Slattery SD, Mancini MA, Brinkley BR, Hall RM. *Cell Cycle*. 2009; 8:2986–2997.
17. Tsai MY, Wiese C, Cao K, Martin O, Donovan P, Ruderman J, Prigent C, Zheng Y. *Nature Cell Biology*. 2003; 5:242–248.
18. Eyers P, Erikson E, Chen L, Maller J. *Current Biology*. 2003; 13:691–697. [PubMed: 12699628]
19. Kufer TA, Silljé HHW, Körner R, Gruss OJ, Meraldi P, Nigg EA. *J. Cell Biol*. 2002; 158:617–623. [PubMed: 12177045]
20. Wittmann T, Wilm M, Karsenti E, Vernos I. *J. Cell Biol*. 2000; 149:1405–1418. [PubMed: 10871281]
21. Bayliss R, Sardon T, Vernos I, Conti E. *Molecular Cell*. 2003; 12:851–862. [PubMed: 14580337]
22. Terada Y. *Cell Struct. Funct*. 2001; 26:653–657. [PubMed: 11942622]
23. Honda R, Körner R, Nigg E. *Molecular Biology of the Cell (Formerly: Cell Regulation)*. 2003; 14:3325–3341.
24. Bishop J, Schumacher J. *Journal of Biological Chemistry*. 2002; 277:27577–27580. [PubMed: 12048181]
25. Jelluma N, Brenkman AB, van den Broek NJF, Crujisen CWA, van Osch MHJ, Lens S, Medema RH, Kops GJPL. *Cell*. 2008; 132:233–246. [PubMed: 18243099]
26. Han Z, Riefler G, Saam J, Mango S, Schumacher J. *Current Biology*. 2005; 15:894–904. [PubMed: 15916946]
27. Zachos G, Black EJ, Walker M, Scott MT, Vagnarelli P, Earnshaw WC, Gillespie DA. *Developmental Cell*. 2007; 12:247–260. [PubMed: 17276342]
28. Petsalaki E, Akoumianaki T, Black E, Gillespie D, Zachos G. *Journal of Cell Biology*. 2011; 195:449–466. [PubMed: 22024163]
29. Sun L, Gao J, Dong X, Liu M, Li D, Shi X, Dong J, Lu X, Liu C, Zhou J. *Proceedings of the National Academy of Sciences - PNAS*. 2008; 105:7153–7158.
30. Rosasco-Nitcher S, Lan W, Khorasanizadeh S, Stukenberg PT. *Science*. 2008; 319:469–472. [PubMed: 18218899]
31. Sessa F, Mapelli M, Ciferri C, Tarricone C, Areces L, Schneider T, Stukenberg PT, Musacchio A. *Molecular Cell*. 2005; 18:379–391. [PubMed: 15866179]
32. Yasui Y, Urano T, Kawajiri A, Nagata K, Tatsuka M, Saya H, Furukawa K, Takahashi T, Izawa I, Inagaki M. *Journal of Biological Chemistry*. 2003; 279:12997–13003. [PubMed: 14722118]
33. Bolton M, Lan W, Powers S, McClelland M, Kuang J, Stukenberg T. *Molecular Biology of the Cell (Formerly: Cell Regulation)*. 2002; 13:3064–3077.
34. Chen J, Jin S, Tahir S, Zhang H, Liu X, Sarthy A, McGonigal P, Liu Z, Rosenberg S, Ng S. *Journal of Biological Chemistry*. 2002; 278:486–490. [PubMed: 12419797]
35. Kelly A, Ghenoiu C, Xue J, Zierhut C, Kimura H, Funabiki H. *Science*. 2010; 330:235–239. [PubMed: 20705815]
36. Chu Y, Yao PY, Wang W, Wang D, Wang Z, Zhang L, Huang Y, Ke Y, Ding X, Yao X. *Journal of Molecular Cell Biology*. 2011; 3:260–267. [PubMed: 21148584]

37. Jacob N, Cooley, Shirai, Chakravarti. *Oncotargets and Therapy*. 2012; 5:7. [PubMed: 22375097]
38. Murata-Hori M, Tatsuka M, Wang Y. *Molecular Biology of the Cell (Formerly: Cell Regulation)*. 2002; 13:1099–1108.
39. Tseng BS, Tan L, Kapoor TM, Funabiki H. *Developmental Cell*. 2010; 18:903–912. [PubMed: 20627073]
40. Terada Y, Tatsuka M, Suzuki F, Yasuda Y, Fujita S, Otsu M. *EMBO Journal, The*. 1998; 17:667–676.
41. Bird A, Hyman A. *Journal of Cell Biology*. 2008; 182:289–300. [PubMed: 18663142]
42. Abramoff MD, Magelhaes PJ, Ram SJ. *Biophotonics International*. 2004; 11:36–42.
43. Hsu J, Sun Z, Li X, Reuben M, Tatchell K, Bishop D, Grushcow J, Brame C, Caldwell J, Hunt D, Lin R, Smith M, Allis C. *Cell*. 2000; 102:279–291. [PubMed: 10975519]
44. Murnion M, Adams R, Callister D, Allis C, Earnshaw W, Swedlow J. *Journal of Biological Chemistry*. 2001; 276:26656–26665. [PubMed: 11350965]
45. Giet R, Glover D. *Journal of Cell Biology*. 2001; 152:669–682. [PubMed: 11266459]
46. Ota T, Suto S, Katayama H, Han Z, Suzuki F, Maeda M, Tanino M, Terada Y, Tatsuka M. *Cancer Research*. 2002; 62:5168–5177. [PubMed: 12234980]
47. Crosio C, Fimia G, Loury R, Kimura M, Okano Y, Zhou H, Sen S, Allis C, Sassone-Corsi P. *Molecular and Cellular Biology*. 2002; 22:874–885. [PubMed: 11784863]
48. Ditchfield C, Johnson VL, Tighe A, Ellston R, Haworth C, Johnson T, Mortlock A, Keen N, Taylor SS. *J. Cell Biol*. 2003; 161:267–280. [PubMed: 12719470]
49. Gassmann R, Carvalho A, Henzing AJ, Ruchaud S, Hudson DF, Honda R, Nigg EA, Gerloff DL, Earnshaw WC. *J. Cell Biol*. 2004; 166:179–191. [PubMed: 15249581]
50. Jeyapakash A, Klein U, Lindner D, Ebert J, Nigg E, Conti E. *Cell*. 2007; 131:271–285. [PubMed: 17956729]
51. Aguirre-Portoles C, Bird AW, Hyman A, Canamero M, Perez de Castro I, Malumbres M. *Cancer Research*. 2012

Highlights

- TPX2 acts as a co-activator protein for Aurora B kinase
- TPX2 serves as a scaffold protein for assembly of the Chromosomal Passenger Complex
- Overexpression of TPX2 B causes mitotic defects that reflect Aurora B inhibition
- The levels of TPX2 correlate with Aurora B activity in pancreatic cancer cells

Fig 1. TPX2 interacts with Chromosomal Passenger Complex (CPC) proteins in *Xenopus* egg extracts (XEE)

(A) and (B) GST IPs were performed in M-phase XEE in presence of either GST or GST-FL TPX2. This was followed by western blotting of the immunoprecipitates. (A) A GST blot showing successful pulldown of GST and GST-TPX2 (arrows). Additional bands in the GST-FL TPX2 lane represent the degradation products of the purified GST-FL TPX2 protein. (B) GST-FL TPX2 specifically associates with the CPC proteins Aurora B, INCENP and Survivin in XEE. A blot for Aurora A is included as a positive control for the GST-TPX2 IP.

Fig 2. Depletion of endogenous TPX2 protein reduces Aurora B activity in XEE

Western blot analysis of the samples is presented. (A) TPX2 was immunodepleted from XEE using the protocol from (Wittman et. al, 2000) [20]. Either the mock (IgG)-depleted or TPX2-depleted egg extracts were then used to perform Aurora B IPs. The left panel shows the levels of proteins in the mock and TPX2-depleted egg extracts. The right panel exhibits that the depletion of endogenous TPX2 causes a decrease in Aurora B activation and also leads to a reduction in Survivin-Aurora B and INCENP-Aurora B interactions. (B) Quantification of the western blot signal for phospho-Aurora B in the Aurora B IPs from three independent experiments of panel (A). (C) TPX2 was again immunodepleted from XEE and Aurora B IPs were performed as mentioned earlier. Further, the TPX2

immunodepleted XEEs were reconstituted with recombinant, bacterially expressed and purified GST-TPX2. The left panel shows inputs for the different egg extracts. The right panel exhibits that the addition of recombinant GST-TPX2 can completely rescue the decrease in Aurora B activity and the reduction in Survivin-Aurora B and INCENP-Aurora B interactions that is mediated by the depletion of endogenous TPX2. (D), (E):

Densitometric quantification of western blot data from three independent experiments of panel (A) is presented. (D) The depletion of TPX2 from XEE decreases the association of Aurora B with Survivin in Aurora B IPs. (E) A decrease in the association of Aurora B with INCENP upon the depletion of TPX2 is seen in Aurora B IPs in XEE, albeit weaker than that seen in panel (D).

Fig 3. Addition of an excess of FL-TPX2 protein and its truncation TPX2 B decreases Aurora B activity in XEE

(A) Diagram representing the different GST-tagged *Xenopus* TPX2 truncation constructs that were generated. (B), (C), (D), (E), (F), (G), (H): Aurora B warm IPs were performed in the presence of over a 20-fold excess of either GST (control), GST-FL TPX2 or GST-TPX2 truncation proteins. (B) Western blot demonstrating the strength of the interaction between FL-TPX2 or its different truncations and Aurora B in Aurora B IPs. Both FL-TPX2 and TPX2 B can interact the strongest with Aurora B. TPX2 A, the Aurora A-interacting region of TPX2 interacts the weakest with Aurora B in XEE. The line indicates that the blot was cropped to eliminate a non-relevant lane. (C), (E) Western blot analysis of the respective

Aurora B IP samples is presented. (C) An excess of both FL TPX2 and TPX2 B leads to the inactivation of Aurora B in Aurora B IPs. (D) Quantification of the western blot signal for phospho-Aurora B in Aurora B IPs from three independent experiments of panel (B). (E) An excess of GST-TPX2 B is sufficient to dissociate both Survivin and INCENP from Aurora B in an Aurora B IP. (F) Quantification of the western blot signal for phospho-Aurora B in Aurora B IPs from three independent experiments of panel (E). (G), (H): Densitometric quantification of western blot data from three independent experiments of panel (C) is presented. (G) Adding an excess of FL-TPX2, TPX2 A and TPX2 B proteins causes a decrease in Aurora B activity in Aurora B IPs in XEE. (H) Of all the truncation proteins of TPX2, addition of an excess of only FL-TPX2 and TPX2 B can reduce the association of Survivin with Aurora B in Aurora B IPs in XEE. (I) Western blot analysis of a MT pelleting assay showing that over a 20-fold excess of both TPX2 B and TPX2 D can reduce the amount of active Aurora B that is present on MTs at 15 minutes (middle panel). The tubulin blot demonstrates that an equal amount of pelleted MTs were loaded onto all the lanes for the western blot (lowest panel).

Fig 4. TPX2 B is sufficient to enhance Survivin-Aurora B interaction and Aurora B kinase activity *in vitro*

AP= Alkaline phosphatase (A) Aurora B and Survivin proteins were either mixed alone or in the presence of 1, 5 or 30 μ g of recombinant myc-TPX2 B protein and Aurora B IPs were performed. Western blotting of the IP samples indicates that addition of both 5 and 30 μ g of myc-TPX2 B increases the association of Aurora B with Survivin *in vitro*. The negative controls (Aurora B antibody (Aur B Ab)-conjugated beads with Survivin and myc-TPX2 B proteins but lacking Aurora B protein) do not show any non-specific interaction of Survivin with the beads in the presence of myc-TPX2 B. (B) An *in vitro* Aurora B kinase assay was performed either in presence or in the absence of myc-TPX2 B and the reaction was

terminated after 5 or 10 minutes. Following this, the samples were subjected to western blot analysis. Addition of myc TPX2 B enhances the phosphorylation of Histone H3 Serine 10 (HH3 Ser 10) by Aurora B at both 5 and 10 minutes.

Fig 5. TPX2 and CPC interact and co-localize in human cell lines

(A), (B) and (C): Western blot analysis of the immunoprecipitates is presented. (A) IgG or Aurora B IPs were performed in nocodazole-synchronized 293T cells. TPX2 interacts with Aurora B under endogenous conditions. The dotted lines indicate cropping of the blot to remove empty lanes. The INCENP blot is a positive control for the Aurora B IP. (B), (C) Myc IPs were performed in nocodazole-synchronized mitotic HeLa cells. (B) Myc-FL TPX2 efficiently associates with Aurora B, INCENP and Survivin in mitotic HeLa cells. (C) Myc-TPX2 B specifically associates with Aurora B, Survivin and INCENP in nocodazole-synchronized HeLa cells. (D), (E) and (F): Immunofluorescence analysis of endogenous proteins is presented. The upper, middle and lower panels of each sub-figure represent a cell

in prophase/prometaphase, metaphase and telophase respectively. The white arrows highlight the areas of co-localization of TPX2 with the different CPC proteins. The DNA is stained blue using DAPI. (D) Aurora B (red) co-localizes with TPX2 (green) at the kinetochore MTs, spindle poles and midbody during prometaphase, metaphase and telophase, respectively. (E) Survivin (red) co-localizes with TPX2 (green) at the kinetochore MTs, spindle poles and midbody during prophase, metaphase and telophase, respectively. (F) INCENP (red) co-localizes with TPX2 (green) at the kinetochore MTs, spindle poles and midbody during prophase, metaphase and telophase, respectively.

Fig 6. Over-expression of mCherry-TPX2 B causes mitotic abnormalities in HeLa cells
 (A) Immunofluorescence (IF) images demonstrating that the transfection of HeLa cells with mCherry-TPX2 B (red) causes severe defects in chromosome alignment during metaphase. The DNA is stained blue with DAPI and the microtubules are stained green using an anti- α -tubulin antibody. The bar size is 5 μ m. (B) Quantification of the metaphase chromosome alignment defects from three independent experiments. 200 total cells were counted and scored for both the mock-transfected (mCherry-transfected) and mCherry-TPX2B transfected samples. (C) IF images demonstrating that the transfection of HeLa cells with mCherry-TPX2 B (red) causes an accumulation of INCENP (green) at the spindle poles (white arrows middle and lower panels) and results in diffused INCENP staining on the DNA (blue) during metaphase. Very high levels of mCherry-TPX2 B cause almost complete displacement of INCENP from the DNA (lowest panel). The bar size is 5 μ m. (D) Quantification of the INCENP localization abnormalities from three independent experiments. 148 mock-transfected cells and 147 mCherry-TPX2 B transfected cells were counted and scored for metaphase INCENP localization abnormalities.

Fig 7. Interaction with TPX2 correlates with increased Aurora B activity in pancreatic cancer cells

Aurora B IPs were performed in nocodazole-synchronized Panc-1 and CFPAC-1 pancreatic cancer cell lines. (A) Western blot analysis of the input showing the endogenous levels of different proteins in the cell lysates. (B) Western blot analysis of Aurora B IP samples demonstrating that an increased TPX2 association with Aurora B in the CFPAC-1 cell line enhances Aurora B-Survivin and Aurora B-INCENP interactions in this cell line. This results in increased Aurora B activation in this cell line. The asterisk denotes non-specific background.

Fig 8. Model showing the mechanism of Aurora B activation by TPX2

This cartoon depicts the manner by which TPX2 activates Aurora B. Addition of TPX2 to TPX2-depleted egg extract increases Aurora B activity by enhancing its interaction with other CPC proteins. However, an excess of TPX2 sequesters Aurora B activators away from Aurora B, ultimately leading to its decreased activation. The size of the phosphorylation mark on Aurora B denotes the extent of Aurora B activation. Different colored circles denote distinct proteins/protein complexes. AurB = Aurora B, Surv = Survivin, Bor = Borealin. The sizes of proteins are not to scale.