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Tumor and Stem Cell Biology

Tpx2 Controls Spindle Integrity, Genome Stability, and Tumor Development

Cristina Aguirre-Portolés1, Alexander W. Bird2, Anthony Hyman3, Marta Cañamero2, Ignacio Pérez de Castro1, and Marcos Malumbres1

Abstract

Tpx2 is a microtubule-associated protein that activates the cell-cycle kinase Aurora A and regulates the mitotic spindle. Overexpression of Tpx2 is associated with the development of different human tumors and strongly correlates with chromosomal instability. By analyzing a conditional null mutation in the mouse Tpx2 gene, we show here that Tpx2 expression is essential for spindle function and chromosome segregation in the mouse embryo. Conditional genetic ablation of Tpx2 in primary cultures resulted in deficient microtubule nucleation from DNA and aberrant spindles during prometaphase. These cells eventually exited from mitosis without chromosome segregation. In addition, Tpx2 haploinsufficiency led to the accumulation of aneuploidies in vivo and increased susceptibility to spontaneous lymphomas and lung tumors. Together, our findings indicate that Tpx2 is essential for maintaining genomic stability through its role in spindle regulation. Subtle changes in Tpx2 expression may favor tumor development in vivo. Cancer Res; 72(6); 1518–28. ©2012 AACR.

Introduction

Formation of a proper bipolar spindle is an essential process required for accurate chromosome segregation. This dynamic microtubule-based structure is responsible for chromosomal movements that ensure the equal distribution of a single, complete complement of the genome into 2 daughter cells. Chromatin-driven microtubule nucleation and organization depends on a gradient of Ran-GTP known to promote microtubule nucleation and stabilization surrounding the chromosomes (1). Tpx2 is one of the best studied factors regulated by the Ran-GTP gradient during mitosis. First described by Wittmann and colleagues in 1998 as the Targeting Protein for Xklp2 in Xenopus laevis (2), this protein regulates the spindle formation at different levels. Once released from its inhibitory complex with importin-α, Tpx2 is able to promote microtubule nucleation from the chromatin, both in Xenopus extracts and in human cells (3–5). Tpx2 recruits Aurora A kinase to microtubules (6) and drives the activation of this kinase during mitosis (7, 8). The N-terminal domain of Tpx2 interacts with Aurora A protecting the Thr288 in the T-loop of the kinase from dephosphorylation by the Phosphatase Protein 1 (PP1; refs. 7, 9). Cells deficient in the Aurora A/Tpx2 complex present short spindles which, despite of being able to segregate chromosomes, result in mitotic failure (4).

Alterations in the expression levels of several mitotic regulators have been associated with tumor formation in humans (reviewed in refs. 10, 11). In fact, either upregulation or downregulation of several mitotic regulators may lead to spontaneous tumor formation through an increase in aneuploidy (11, 12). For instance, both downregulation and overexpression of Mad2 (also called MAD2L1) result in increased susceptibility to tumor development (13, 14). Aurora A is also overexpressed in various cancers including breast, colorectal, and bladder tumors (10), and its partial deficiency results in pulmonary carcinomas, squamous cell carcinoma, and lymphomas in Aurora A heterozygous mice (15). Tpx2 is also highly expressed in human tumors (reviewed in refs. 10, 16) and overexpression of Tpx2 displays the highest correlation with the presence of chromosomal instability (CIN) in several human cancers (17).

To understand the physiologic relevance of Tpx2, we have generated Tpx2-deficient embryos and mice. Complete genetic ablation of Tpx2 results in defective microtubule nucleation and spindle abnormalities that prevent chromosome segregation in early embryos or cultured fibroblasts. Partial deficiency in Tpx2 levels results in the accumulation of aneuploidies in vitro and in vivo and shorter lifespan due to spontaneous tumor development, suggesting that the control of spindle integrity by Tpx2 prevents genomic instability in vivo.

Materials and Methods

Generation and characterization of Tpx2 mutant mice

The ES cell clone D028B03 was obtained from the Gene Trap German Consortium (GGTC). The exact position of the
insertion was determined by PCR and DNA sequencing (Fig. 1). Chimeras were generated through standard blastocyst microinjection. Tpx2 heterozygous mice were obtained by intercrossing with C57BL/6J wild-type mice. Genotyping of Tpx2 alleles (wild type, trapped allele, conditional, and knockout) was done by PCR using the following oligonucleotides: wild-type allele (+): Tpx2_D028B03_F2: 5′-GAA-TTCAGAGGCCCAGACAG-3; Tpx2_D028B03_R2: 5′-GAGTTCCAGGACAGCCAGAG-3; trapped allele (−): Tpx2_D028B03_F2: 5′-GAATTCAGAGGCCCAGACAG-3; Tpx2_D028B03_R3: 5′-CTGCCGAAAAGCGCTTCTA-3; conditional allele (lox): Tpx2_D028B03_F3: 5′-TTCTCTGCATAGCCAGGT-3; Tpx2_D028B03_R3: 5′-GGAGGATGGGAAAGACAT-3; knockout allele (Δ): Tpx2_D028B03_F3: 5′-TTCTCTGCATAGCCAGGT-3; B32: 5′-CAAGGCGGTAATTGGGTAACG-3.

Mice were housed at the pathogen-free animal facility of the Centro Nacional de Investigaciones Oncológicas (Madrid) following the animal care standards of the institution. These animals were observed daily and sick mice were euthanized humanely in accordance with the Guidelines for Humane End Points for Animals used in biomedical research. For histologic studies, dissected organs were fixed in 10% buffered formalin (Sigma) and embedded in paraffin wax. Sections of 3- or 5-μm thickness were stained with hematoxylin and eosin (H&E).

**Early embryo culture and immunofluorescence**

Fertilized embryos were collected by flushing the oviduct through the infundibulum of super-ovulated females from crosses between Tpx2<sup>+/−</sup> mice, with HEPES-buffered Medium 2 (M2; Sigma) at embryonic day (E)1.5. Embryos were individually cultured in vitro in potassium simplex optimized medium (KSO; Chemicon International Inc.) and photographed daily for up to 4 days. PCR analysis of embryos was done as described previously (18). For immunofluorescence analysis, embryos were fixed with cold methanol during 1 hour at −20°C, washed in PBS containing 0.1% bovine serum albumin (BSA; Sigma) and incubated with 0.1% Triton X-100. Embryos were then blocked with 10% of serum in PBS-0.1% BSA and incubated with the following primary antibodies: α-tubulin (mouse monoclonal, clone DM1a, 1:2,000; Sigma), Anti-Centromere Antibody (ACA, 1:100; Antibodies Inc.), and Tpx2 (Rabbit polyclonal, 1:200, Lifespan) for 2 hours at 37°C. The matching secondary antibodies, with different Alexa dyes (488, 594, and 647, 1:1,000), were from Molecular Probes (1:250; Invitrogen). Secondary antibodies (Alexa 488, 594, or 647; 1:1,000) were incubated for 1 hour at room temperature. Secondary antibodies (Alexa 488, 594, or 647; 1:1,000) were incubated for 1 hour at room temperature. Secondary antibodies (Alexa 488, 594, or 647; 1:1,000) were incubated for 1 hour at room temperature. Secondary antibodies (Alexa 488, 594, or 647; 1:1,000) were incubated for 1 hour at room temperature. Secondary antibodies (Alexa 488, 594, or 647; 1:1,000) were incubated for 1 hour at room temperature. Secondary antibodies (Alexa 488, 594, or 647; 1:1,000) were incubated for 1 hour at room temperature. Secondary antibodies (Alexa 488, 594, or 647; 1:1,000) were incubated for 1 hour at room temperature. Secondary antibodies (Alexa 488, 594, or 647; 1:1,000) were incubated for 1 hour at room temperature. Images were obtained using a confocal ultraspectral microscope (Leica TCS-SP2-AOBS-UV) or confocal ultraspectral microscope Leica TCS-SP5.

**Mouse embryonic fibroblast culture, immunofluorescence, and citometry**

Mouse embryonic fibroblasts (MEF) were generated from E13.5 embryos and cultured using standard protocols (19). Adenoviruses expressing Flp or Cre (supplied by the Iowa University) and siRNAs against Mad2 (Dharmacon) were used following the manufacturer’s recommendations. Briefly, adenoviruses were transduced in confluence and low serum conditions and, 48 hours later, cells were split and nucleofected with the corresponding siRNA. For immunofluorescence, cells were rinsed with PBS and fixed in 4% PFA-PBS for 7 minutes and then left in cold methanol overnight at −20°C. Cells were stained with antibodies against α-tubulin (mouse monoclonal, clone DM1a, 1:1,000; Sigma), CREST (1:500; A. Hyman’s lab), Eg5 (kindly provided by Dr. Yannick Arlot-Bonnemains; rabbit polyclonal 1:250), Kif15 (kindly provided by Isabelle Vernos; rabbit polyclonal 1:250), Tpx2 (rabbit polyclonal, 1:500, Lifespan; or rabbit polyclonal from A. Hyman’s lab; 1:500), Cep135 (1:500; A. Hyman’s lab), γ-tubulin (mouse monoclonal, clone GTU88, 1:1,000, Sigma), or Aurora A (mouse monoclonal, clone 35C1, 1:500; Abcam) for 1 hour at room temperature. Secondary antibodies (Alexa 488, 594, or 647; 1:1,000) were incubated for 1 hour at room temperature. Images were taken using a Leica D3000 microscope or confocal ultraspectral microscope Leica TCS-SP5. For DNA content analysis, cells were fixed in 70% ethanol overnight

![Figure 1](https://example.com/image1.png)
at ~20°C and stained with propidium iodide (20 μg/mL; Sigma) in presence of RNase A (0.2 mg/mL; Qiagen) for 30 minutes at 4°C and then analyzed by flow cytometry (Becton-Dickinson).

Biochemical analysis and reverse transcriptase PCR
For immunoblotting, cells were harvested and lysed with radioimmunoprecipitation assay buffer and 50 μg of total protein was separated by SDS-PAGE and probed with antibodies against Tpx2 (rabbit polyclonal, 1:500, A. Hyman’s lab), Mad2 (Mouse monoclonal, 1:500, MBL International) or β-actin (mouse monoclonal 1:2,000, Abcam). For quantitative reverse transcriptase PCR (RT-PCR), TRI Reagent (Invitrogen) and the SuperScript III Platinum One-Step qRT-PCR Kit were used as recommended by the manufacturers. The sequence of the specific primers used for Tpx2 is available under request to the authors.

Time-lapse imaging and microtubule growth assays
MEFs were transduced with a lentiviral vector expressing H2B protein fused to mRFP (pHIV-H2BmRFP; ref. 20). Forty-eight hours after serum stimulation (15% FBS) of quiescent cells, time-lapse images were acquired using a DeltaVision RT imaging system (Applied Precision, LLC; IX70/71; Olympus) equipped with a charge-coupled device camera (CoolSNAP HQ; Roper Scientific). Images were acquired at 10 minutes intervals using a 20X PlanApo N 1.35 NA UPLanApo objective in the DeltaVision equipment (Applied Precision, LLC) software.

Metaphase spreads
Cells were hypotonically swollen in 40% full medium, 60% tap water for 5.5 minutes. Hypotonic treatment was stopped by adding an equal volume of Carnoy’s solution (75% pure methanol, 25% glacial acetic acid), cells were then spun down, and resuspended and fixed with Carnoy’s solution for 10 minutes. After fixation, cells were dropped from a 5-cm height onto glass slides previously treated with 4% acetic acid. Slides were mounted with ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) and images were acquired with a Leica D3000 microscope and a 60X PlanApo N 1.42 NA objective. Chromosomes from 30 cells per genotype were counted using ImageJ software.

Results
Tpx2 is required for cell division during early embryonic development
To study the physiologic functions of Tpx2 in vivo we used a conditional gene trap allele as represented in Fig. 1A (see Methods). This strategy consists on the insertion of a β-geo (β-galactosidase plus neo resistance) cassette that carries a splicing acceptor (SA) and a poly(A) sequence for termination of transcription. Alternated and inverted wild-type or mutant fl and loxP sites flanking the resistance cassette allow its inversion in the presence of Flp or Cre recombinases (21), allowing the switching from a conditional allele Tpx2(lox) to the null allele Tpx2(Δ), in which the Tpx2 transcript is trapped by the SA-β-geo cassette (Fig. 1A). Both the Tpx2(−) and the Tpx2(Δ) are null alleles and led to similar phenotypes in homozygosity.

No homozygous Tpx2<sup>−/−</sup> mutant was born from intercrosses between Tpx2<sup>+/−</sup> mice and, similarly, null embryos were absent between embryonic day (E)8.5 and E17.5 (Fig. 1B), suggesting early embryonic lethality in the absence of Tpx2. We then harvested embryos at E1.5 and kept them in culture following its development by bright field microscopy. E1.5 Tpx2<sup>−/−</sup> embryos were observed at the expected Mendelian ratio (23%; N = 97). However, whereas Tpx2<sup>+/−</sup> and Tpx2<sup>+/+</sup> embryos were capable of generating normal blastocysts, Tpx2 null embryos got arrested at the morula stage (Fig. 1C), suggesting that Tpx2 is essential for preimplantation development of the mouse.

To characterize the origin of the early lethality, we carried out immunofluorescence 1 (early morula), 2 (compacted morula), or 3 (blastocyst) days after extracting the embryos at E1.5. Tpx2 null cells were not able to establish a proper bipolar spindle in early morulas and these cells presented collapsed or monopolar spindles (Fig. 2A and B). At this stage, we observed the absence of tetraploid cells that suggested defective chromosome segregation (Fig. 2A). Although the structure of the spindle was disturbed, no differences in the intensity of α-tubulin were observed when comparing wild-type bipolar spindles with the aberrant spindles observed in Tpx2<sup>−/−</sup> cells (P = 0.8882; Fig. 2B). After 2 days in culture, wild-type embryos presented 18 cells per morula, whereas the number of cells in mutant embryos varied from 9 to 13 (P < 0.0001; Fig. 2C). Tpx2 null cells presented a statistically significant increase in the volume of the nuclei (P < 0.002; Fig. 2C), suggesting lack of proper cell division. After 3 days in culture, Tpx2<sup>−/−</sup> embryos remained arrested at the morula stage, whereas wild-type embryos had formed early blastocysts and presented normal bipolar spindles in mitotic cells (Fig. 2D). These results indicated that Tpx2 is required for the formation of normal bipolar spindles and chromosome segregation during early embryonic development.

Defective cell-cycle progression and chromosome segregation in Tpx2 null MEFs
We took advantage of the possibility to abrogate the expression of Tpx2 in a conditional manner under Cre recombinase expression (Fig. 1A). We derived Tpx2<sup>lox/lox</sup> MEFs from E13.5...
embryos and these cells were transduced with Cre expressing adenoviruses to genetically abrogate the expression of Tpx2 (Tpx2\(^{fl/fl}\)). Flp recombinase was used as a control with no effect in Tpx2\(^{lox/lox}\) alleles. Tpx2\(^{fl/fl}\) cultures showed a severe impairment in their proliferation capacity when compared with control MEFs (\(P = 0.0026; \text{Fig. 3A})\). We then induced the genetic ablation of Tpx2 in serum-starved cells and followed cell-cycle progression by different techniques after the addition of serum (Fig. 3B). Control cells completed a normal cell cycle in about 28 hours, whereas Tpx2 null cells accumulated as 4N cells 28 or 32 hours after entering into the cell cycle. We also detected an increase in the percentage of Tpx2\(^{fl/fl}\) cells with more than 4N DNA content (13.0% in Tpx2\(^{fl/fl}\) vs. 7.5% in Tpx2\(^{lox/lox}\) at 24 hours; \(P = 0.0061; \text{Fig. 3B})\). In addition, we observed a statistically significant increase in the sub-G0 (less than 2N) population in Tpx2-deficient MEFs suggesting cell death in this population (Fig. 3B). Tpx2-deficient cultures also contained binucleated cells (Fig. 3C) as well as aberrant cells with multiple nuclei or micronuclei (Fig. 3C and D). All these phenotypes suggested abnormal chromosome segregation and the presence of aberrant ploidy in Tpx2 null cells.

We next studied the onset and exit from mitosis following individual, synchronized cells in a time-lapse imaging experiment. To visualize chromatin, cells were transduced with a construct expressing histone H2B fused to mRFP (red fluorescence). The percentage of cells entering in mitosis was similar in Tpx2\(^{lox/lox}\) (26.2 ± 7.0%) and Tpx2\(^{fl/fl}\) (22.4 ± 6.8%) MEFs (\(P = 0.7315; \text{Fig. 4A})\). Control Tpx2\(^{lox/lox}\) MEFs were able to carry out mitosis in an average time of 73 minutes (73.1 ± 3.2; \(N = 52; \text{Fig. 4B})\). In contrast, Tpx2\(^{fl/fl}\) cells stayed in mitosis for an average time of 122 minutes (121.5 ± 12.4; \(N = 39\)) and exited mitosis without chromosome segregation and as a single cell in most cases (96.9%; \(\text{Fig. 4A})\). A significant number of cells died in mitosis or during interphase after the first mitosis in agreement with the increase in the sub-G0 population observed in previous assays (Fig. 3B). Tpx2\(^{+/+}\) cells presented no alteration in cell proliferation, mitotic progression, or nuclear...
morphology when compared with wild-type cultures (Supplementary Fig. S1).

The spindle assembly checkpoint (SAC) is the main mechanism that delays mitotic exit until all chromosomes are bioriented in the presence of a normal spindle (for review, see ref. 22). Mad2 is required for monitoring proper chromosome attachment to the spindle microtubules during cell division, and the mitotic delay in presence of microtubule poisons can be overcome by depleting Mad2 from cells (Fig. 4B and C). Similarly, mitotic delay in Tpx2Δ/Δ cells was rescued in the presence of siRNAs against Mad2 (siMad2), and these cells quickly exited mitosis without chromosome segregation [duration of mitosis = 121.5 ± 12.37 minutes in Tpx2Δ/Δ (N = 39) vs. 61.61 ± 3.625 minutes in Tpx2Δ/Δ + siMad2 (N = 31); P < 0.001; Fig. 4B and C]. Cyclin B and securin are normally degraded during mitotic exit in Tpx2 null cells and these mutant cells also display a slow but consistent degradation of cyclin B and securin in the presence of nocodazole (mitotic slippage; data not shown). These data suggested that a SAC-dependent mechanism delays mitotic exit in Tpx2 null cells, probably as a consequence of defects in the proper attachment of chromosomes to the abnormal spindle (see below) in the absence of Tpx2.

**Tpx2 is essential for chromatin-dependent microtubule nucleation**

Tpx2 null cells displayed a significant (P < 0.001) increase in prometaphase figures and the subsequent reduction in the number of metaphase, anaphase, or telophase figures (Fig. 5A), in agreement with the SAC-dependent delay reported above. This prometaphase arrest was associated with a range of spindle abnormalities that could be classified in 4 groups: (i) collapsed spindles, characterized by very robust asters but a decrease in spindle fibers, as well as shorter distances between centrosomes; (ii) weak spindles, characterized by the absence of spindle fibers; as well as (iii) monopolar and (iv)...
multipolar spindles (Fig. 5A). To test whether multipolar spindles were a consequence of overnumerary centrosomes, we used the centriolar marker Cep135, a protein essential for centriole biogenesis (23, 24). Only 2 of the microtubule organizer centers were positive for Cep135 in these abnormal cells (Fig. 5A), although all of them were positive for \( \gamma \)-tubulin (data not shown), confirming that the phenotype was not a result of overnumerary centrosomes. Total \( \alpha \)-tubulin intensity was normal in the aberrant spindles suggesting that microtubule formation itself, and not levels of \( \alpha \)-tubulin, was altered in Tpx2 null cells (Supplementary Fig. S2).

Tpx2 interacts with several molecules, including the kinase Aurora A (6–8), the microtubule-sliding kinesin Eg5 (25, 26), and the plus-end directed kinesin Hklp2/Kif15 (2, 27). We therefore analyzed the status of these proteins in Tpx2-depleted cells. Although Aurora A was not totally displaced from the spindle, we found a significant reduction in the levels of the kinase at spindle microtubules (\( P < 0.0001 \); Fig. 5B). In agreement with data previously reported by 2 different groups (27, 28), the spindle localization of Kif15 was dramatically impaired in the absence of Tpx2 (\( P < 0.0001 \); Fig. 5B). Finally, we found no alterations in centrosome or microtubule
Figure 5. Defective microtubule nucleation in Tpx2 null cells. A, representative images of the spindle abnormalities displayed in Tpx2Δ/Δ cells are shown in the left panels. DAPI was used to stain the DNA, α-tubulin to label the microtubules in red, and Cep135 the centrosome in green. Scale bar, 5 μm. The top histogram shows the distribution of mitotic phases as determined by immunofluorescence. Notice the significant (**, P < 0.001) increase in prometaphase figures. Bottom histogram panel shows the distribution of mitotic aberrations in Tpx2lox/lox and Tpx2Δ/Δ (**, P < 0.05). B, localization of Aurora A (red), Kif15, and Eg5 both in green) in Tpx2 null cells. Scale bars, 5 μm. The fluorescence intensity of Aurora A Kif15 and Eg5 is represented in the histograms (**, P < 0.0001; n.s, P = 1). C, deficient chromatin-dependent microtubule nucleation in Tpx2 null cells 3 minutes (top) after cold treatment. α-Tubulin signals (green) in the vicinity of the chromosomes (DAPI in blue) were used as a marker of chromatin-driven nucleation. In addition, cells were incubated at 37°C during 10 minutes after cold treatment to allow further spindle formation. Quantification of spindle abnormalities is shown in the lower graph. Note that Tpx2Δ/Δ cells were unable to establish robust bipolar spindles after cold treatment. Scale bars, 5 μm. D, Tpx2lox/lox MEFs were incubated on ice for 5 minutes to depolymerize nonstable microtubules (left). The specific localization of Tpx2 at k-fibers was confirmed by kinetochore staining with CREST (red) and anti-Tpx2 antibody (green; left). The stability of Tpx2Δ/Δ k-fibers is defective in the absence of Tpx2 (red; right) as detected by α-tubulin staining (green). CREST is in white and DAPI in blue. Scale bars, 5 μm. The quantification of Tpx2 fluorescence intensity is shown in the right plot (**, P < 0.01).
localization of Eg5 in the aberrant spindles of Tpx2\(^{-/}\) cells (Fig. 5B).

We then analyzed microtubule dynamics by carrying out microtubule repolymerization assays. We ablated Tpx2 expression and promoted depolymerization of microtubules by keeping the culture on ice for 40 minutes following the protocol depicted in Fig. 3B. Repolymerization was analyzed after incubating the cells at 37°C for 3 minutes, to check microtubule nucleation from chromatin, or 10 minutes, to examine centrosome-dependent microtubule growth. As observed after knock down of TPX2 in human cell lines (3, 4), Tpx2 null MEFs presented a clear defect in α-tubulin polymerization from DNA. After 3 minutes at 37°C, short α-tubulin fibers coming from DNA were evident (green signal) in Tpx2\(^{+/}\) cells whereas Tpx2\(^{-/}\) MEFs presented a severe decrease in microtubule formation from chromatin (Fig. 5C). Note that nucleation from centrosomes was not affected as fibers were normally formed from centrosomes. Importantly, spindles in Tpx2\(^{-/}\)MEFs were abnormal after 10 minutes at 37°C, being collapsed or multipolar in 59% and 38% of cases, respectively (Fig. 5C). All these results showed that lack of Tpx2 leads to defective microtubule polymerization from the chromatin resulting in aberrant spindle formation.

The specific localization of Tpx2 and its implication in the stability of k-fibers prompted us to analyze the status of those fibers in Tpx2\(^{-/}\)MEFs. When cells are given cold treatment for short periods of time, the less stable microtubules are depolymerized initially, leaving intact the more stable k-fibers. When the cold treatment is maintained for longer periods, the stability of those k-fibers is also compromised. We treated Tpx2\(^{-/}\)MEFs and Tpx2\(^{+/}\) cells with the kinesin Eg-5 inhibitor monastrol to induce monopolar spindle formation and easily visualize the fibers in the spindles. Tpx2 was specifically localized to k-fibers (Fig. 5D, left panels). By increasing the cold treatment up to 5 minutes, k-fibers were totally lost in Tpx2\(^{-/}\) but not control cells (\(P = 0.0034\); Fig. 5D, right panels), indicating that Tpx2 confers stability to k-fibers possibly facilitating chromosome capture. All these data point out the relevance of Tpx2 in the initial steps of spindle formation and also in the stabilization of the bipolar required for accurate chromosome segregation.

**Increased aneuploidy and tumor formation in the presence of abnormal Tpx2 levels**

Given the requirements for Tpx2 in spindle integrity and genomic stability, we wondered whether Tpx2 haploinsufficiency could lead to accumulation of aneuploidy. Tpx2\(^{-/-}\) mice display a general reduction in Tpx2 mRNA levels both in proliferative (testis, skin, and intestine) and nonproliferative (kidney, brain, and liver) tissues (Supplementary Fig. S3). We extracted splenocytes from Tpx2\(^{-/-}\) mice, stimulated them with mitogens, and carried out karyotype analysis. Sixteen-week-old Tpx2\(^{-/-}\) mice presented higher grade of aneuploidy and 18.3% of cells showed karyotypes that varied from 35 to 80 chromosomes per cell whereas control littermates mice were always 40 or 41 chromosomes per metaphase (Fig. 6A). These control mice always kept 39 to 41 chromosomes even at 90 weeks of age. In contrast, the percentage of aneuploid cells increased to 27% in old Tpx2\(^{-/-}\) mice. These data suggested that Tpx2 plays a critical role in maintaining cellular ploidy of mammals in vivo.

Tpx2\(^{-/-}\) mice, albeit being healthy at birth and totally fertile, showed shorter tumor-free lifespan than their wild-type littermates (Fig. 6B). The histopathologic analysis of the 2 cohorts of Tpx2\(^{-/-}\) (\(n = 14\)) and Tpx2\(^{+/}\) (\(n = 17\)) mice indicated increased development of spontaneous tumors in mutant mice (53% incidence in Tpx2\(^{-/-}\) vs. 7.1% in wild-type mice; \(P < 0.001\)). The most common tumors developed by Tpx2\(^{-/-}\) mice were lymphomas (35.3% of the tumors vs. 7.1% in wild-type mice; Fig. 6B). These mutant lymphomas were not only more abundant but also showed highest aggressiveness as shown by the infiltrations in brain, which are uncommon in other models, and heart among other tissues (Fig. 6C). Tpx2\(^{-/-}\) tumors presented higher levels of aneuploidy (48.9% of aneuploid cells) when compared with Tpx2\(^{+/}\) healthy tissues or wild-type control splenocytes (Fig. 6C).

Tpx2\(^{+/}\) mice also developed frequent lung tumors (alveolar type II cell adenomas and adenocarcinomas; 29.4%; Fig. 6D), as well as other pathologies that were not observed in the wild-type controls, including hyperplasia in the pars intermedia of the pituitary (11.8%; Fig. 6D). All these results indicated that an abnormal expression of Tpx2 can favor tumor development in vivo.

**Discussion**

We report in this article that lack of Tpx2 causes early embryonic lethality during preimplantational development in the mouse. Tpx2 null embryos fail to form blastocysts due to the formation of collapsed spindles and lack of chromosome segregation. Genetic ablation of Eg-5 (kinesin-5), a plus-end-directed motor involved in bipolar spindle formation and maintenance during mitosis, is also associated with early embryonic lethality mainly due to the accumulation of monopolar cells arrested in prometaphase that collapse and are not able to progress beyond the blastocyst stage (29, 30). Interestingly, embryos deficient for Aurora A, probably the main mitotic kinase involved in spindle formation, are also arrested at the same early stages of development (15, 31, 32). Thus, the lack of proteins with major functions in spindle formation and maintenance seems to prevent embryonic development at very early stages.

Tpx2-deficient MEFs require more time than control cells to enter into mitosis (not shown), transiently arrest at prometaphase, and finally exit mitosis without dividing in 2 daughter cells. All these features are also found in Aurora A null primary MEFs (15, 31, 32), which is consistent with the role of Tpx2 as a critical regulator of the Aurora A kinase (4, 6, 8, 33–35). However, some differences are detected between these 2 models suggesting separate functions. For instance, condition-

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major role in centrosome maturation and separation in a Tpx2-independent manner.

Tpx2 is known to regulate the formation of the spindle by promoting microtubule nucleation from the chromatin and stabilizing the spindle microtubules in a Ran-dependent manner (3, 5, 36). In Tpx2 null MEFs, chromosome capture by astral microtubules seems normal. However Tpx2 null mitotic cells are defective in tubulin polymerization from chromatin, which would result in the lack of antiparallel spindle fibers and eventually in the establishment of a weak bipolar spindle. The structural problems displayed by Tpx2 null spindles may result, at least in part, from the absence of essential forces exerted by kinesins such as Eg-5 or Kif15 that ensure spindle elongation. The lack of Kif15 localization in Tpx2 knocked down cells (27) favors this hypothesis. Similarly, the normal microtubule associated localization of Aurora A is lost in Tpx2 null cells (6). The lack of Kif15 localization in Tpx2 knocked down cells (27) favors this hypothesis. Similarly, the normal microtubule associated localization of Aurora A is lost in Tpx2 null cells (6). The relative contribution of these defects to the phenotype observed in the absence of Tpx2 remains elusive, given the technical difficulties of restoring the localization of these proteins in Tpx2 null cells.

Tpx2 is overexpressed in several tumor types such as cervical, lung, and ovarian carcinoma as well as in giant-

Figure 6. Tpx2 heterozygous mice accumulate aneuploid cells and are prone to develop spontaneous lymphomas and adenocarcinomas. A, splenocytes were harvested from Tpx2+/+ and Tpx2+/− mice and kept in culture for 72 hours in the presence of lipopolysaccharide to promote proliferation. Tpx2+/+ mice retain euploid karyotypes whereas Tpx2+/− accumulate aberrations in chromosome inheritance. Chromosomes from 30 cells were counted in each plate. Scale bar, 5 μm. Representative pictures of spleens are shown. Kidney is used as a reference for size. B, tumor-free survival of Tpx2+/+ (N = 14) and Tpx2+/− (N = 17) mice, showing a statistically significant shortening in Tpx2+/− life span (P = 0.0032). The incidence of tumors and the type of pathologies found in these mice is also shown. C, spleen lymphomas in Tpx2+/− mice are characterized by their high-grade aggressiveness including spreading to the heart and brain (H&E staining). Scale bars, 1,000 μm or 200 μm (top right and insets). The ploidy of tumor cells from the spleen in Tpx2+/− mice is also shown and compared with normal, 90-week old splenocytes from wild-type mice. D, representative lung alveolar type II adenocarcinoma (left; scale bars, 500 μm (top) and 200 μm (bottom)) and hyperplasia in pars intermedia of the pituitary gland in Tpx2+/− mice, compared with normal pituitary in control animals (right; scale bars, 200 μm (left) or 100 μm (right)).
cell tumors of bone, where it is amplified (37–40). Moreover, Tpx2 overexpression correlates positively with tumor grade and stage, presence of lymphomatosis, and bad prognosis (41–43). Furthermore, Tpx2 displays the highest correlation with chromosome instability (CIN) among a signature of genes deregulated in human tumors (17). Here, we have shown that genetic ablation of Tpx2 results in chromosome segregation defects and the generation of tetraploid or aneuploid cells. Tpx2+/− mice are also prone to develop a wide spectrum of tumor types, suggesting that this protein may function as a tumor suppressor protecting from genomic instability. A similar increase in the development of spontaneous tumors has been observed in haploinsufficient mouse models of other mitotic genes, including Aurora A, CenpE, Mad2, or Cdh1 (11). Altogether, the fact that overexpression of some of these proteins such as Aurora A, Mad2, or Tpx2 also promotes tumor development in mouse models or correlated with tumor development in human malignancies favor the idea that subtle changes, either up or down, in the level of expression of the same mitotic regulator might have important consequences in genomic instability and cancer development. Tpx2+/− mice accumulate aneuploidy with age indicating that their tissues are genetically unstable. This feature correlates with increased susceptibility to tumor development, supporting the idea that Tpx2 deregulation might eventually act as a driving force of tumor development by the induction of aneuploidy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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