Supplemental Data

Spindle Pole Regulation by a Discrete Eg5-Interacting Domain in TPX2

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Supplemental Results and Discussion

TPX2-CT Does Not Inhibit Exit from Mitosis

Given the observation that embryos injected with TPX2-CT are visually indistinguishable from those arrested in mitosis after injection of the APC/C inhibitor Emi1-CT, we first investigated the possibility that TPX2-CT inhibits the anaphase-promoting complex/cyclosome (APC/C). We coinjected both blastomeres of a two-cell embryo with TPX2 proteins together with radiolabeled *Xenopus* securin, a substrate of the APC/C. The amount of coinjected securin or a nondegradable mutant form (securin DM) alone did not alter cleavage furrow ingression (data not shown). Embryos coinjected with securin and Emi1-CT arrested in mitosis, did not cleave, and showed no securin degradation over long periods of time (Figure S2A). However, in embryos coinjected with TPX2-CT, securin was rapidly degraded, similar to those injected with TPX2-NT. Nevertheless, although embryos injected with Emi1-CT or TPX2-CT could not undergo cleavage, embryos injected with TPX2-NT cleaved normally. Taken together, these results indicate the cleavage arrest induced by TPX2-CT is not a consequence of APC/C inhibition in *Xenopus* embryos.

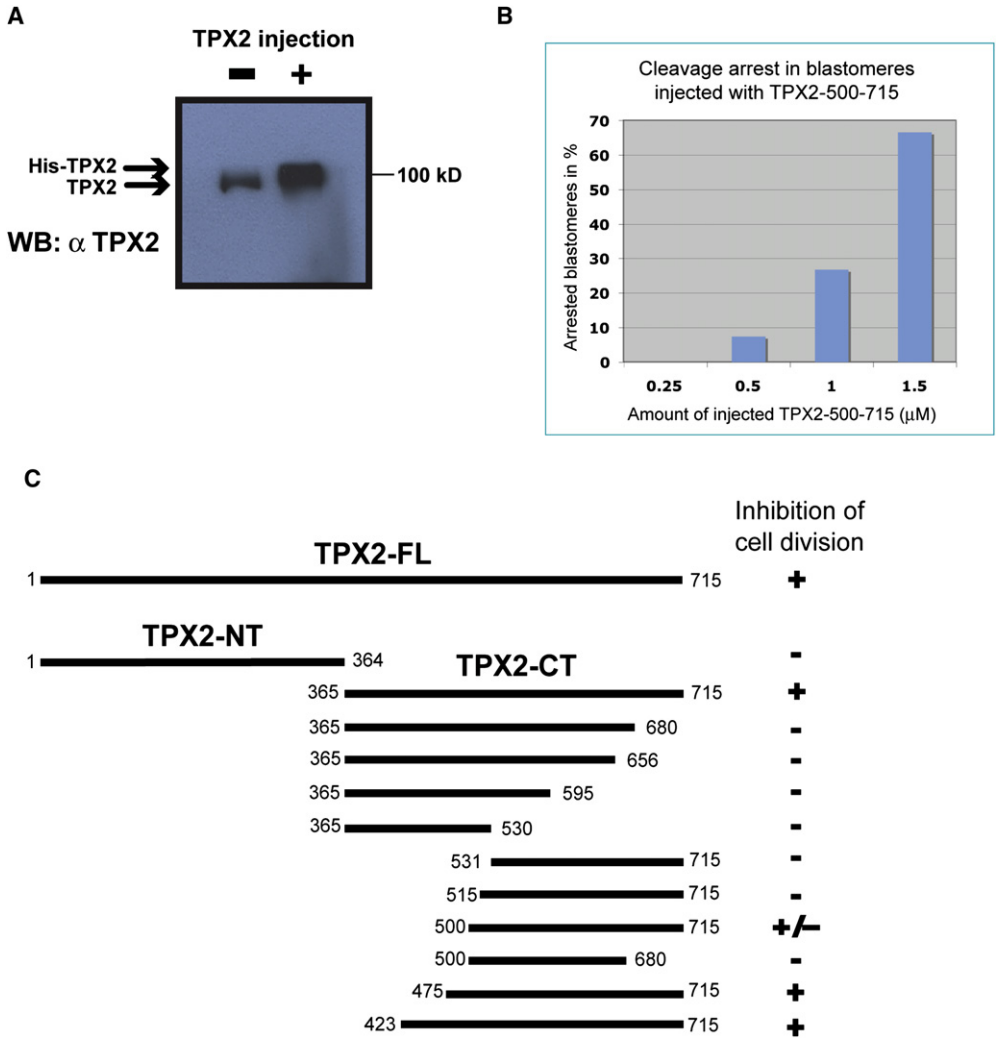


Figure S1. Induction of Cleavage Arrest in Embryos Is Concentration Dependent

(A) TPX2 western blot of uninjected versus TPX2-injected embryos.

(B) Dose-dependent effect of TPX2 (500–715) on embryonic cleavage. Increasing amounts of TPX2 (500–715) were injected into embryos and analyzed for their ability to induce cleavage arrest. The x axis is the final intracellular concentration. For each injected protein concentration, at least 26 embryos were monitored for cleavage defects.

(C) Schematic representation of the indicated TPX2-deletion proteins analyzed for cleavage arrest in *Xenopus* embryos as shown in Figure 1A. Plus (+) indicates constructs induced potent cleavage arrest, whereas minus (−) indicates embryos divided normally.

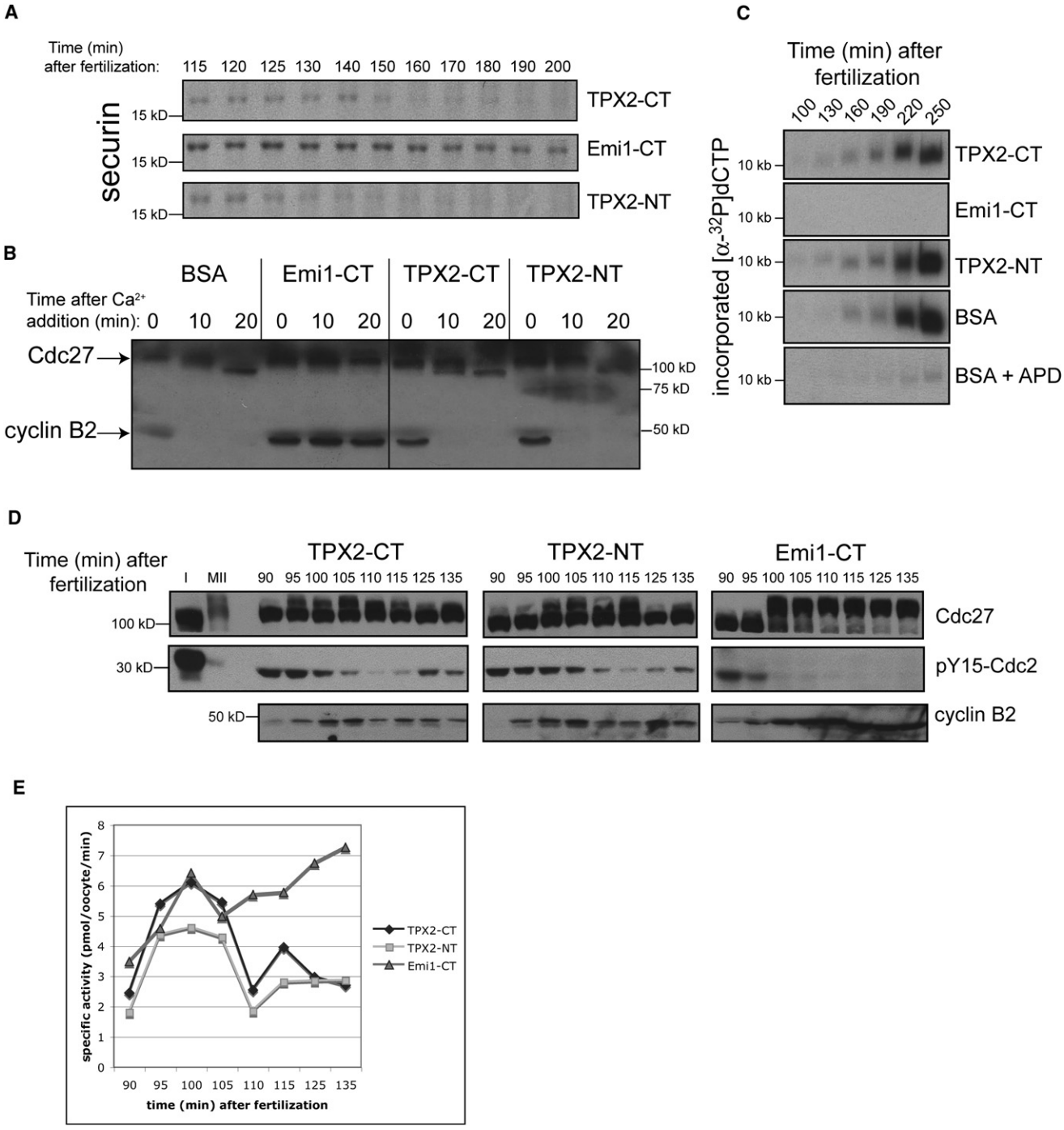


Figure S2. Injection of TPX2-CT Does Not Induce Cell-Cycle Arrest in *Xenopus* Embryos

(A) Analysis of securin degradation in injected embryos. Both blastomeres of two-cell *Xenopus* embryos were coinjected with equal amounts (0.5 μM) of the indicated proteins and 20 nCi of [^{35}S] securin. Securin degradation was monitored in extracts of embryos prepared at the indicated times by SDS-PAGE and autoradiography.

(B) Impact of TPX2 proteins on exit from CSF arrest in egg extracts. CSF-arrested egg extracts were incubated with the indicated proteins and analyzed for Cdc27 phosphorylation and cyclin B2 degradation by western blotting after release into interphase by calcium addition.

(C) Impact of TPX2-proteins on DNA synthesis in embryos. Both blastomeres of two-cell *Xenopus* embryos were coinjected with equal amounts of the indicated proteins and [α-^{32}P]-dCTP. Incorporation of radiolabel was visualized by agarose gel electrophoresis and autoradiography.

(D) Western blot analysis of cell-cycle proteins in embryos after injection of TPX2-proteins. Both blastomeres of two-cell *Xenopus* embryos were injected with equal amounts of TPX2-CT, TPX2-NT, or Emi1-CT. Samples were taken at the indicated time points and subjected to western blotting by using antibodies against Cdc27, pY15-Cdc2, or cyclin B2.

(E) Impact of TPX2-proteins on cell-cycle progression in embryos as measured by histone-H1-directed kinase activity. Both blastomeres of two-cell *Xenopus* embryos were injected with the indicated proteins. Extracts were prepared at the indicated time points and subjected to histone H1 kinase assays as described in the Supplemental Experimental Procedures.

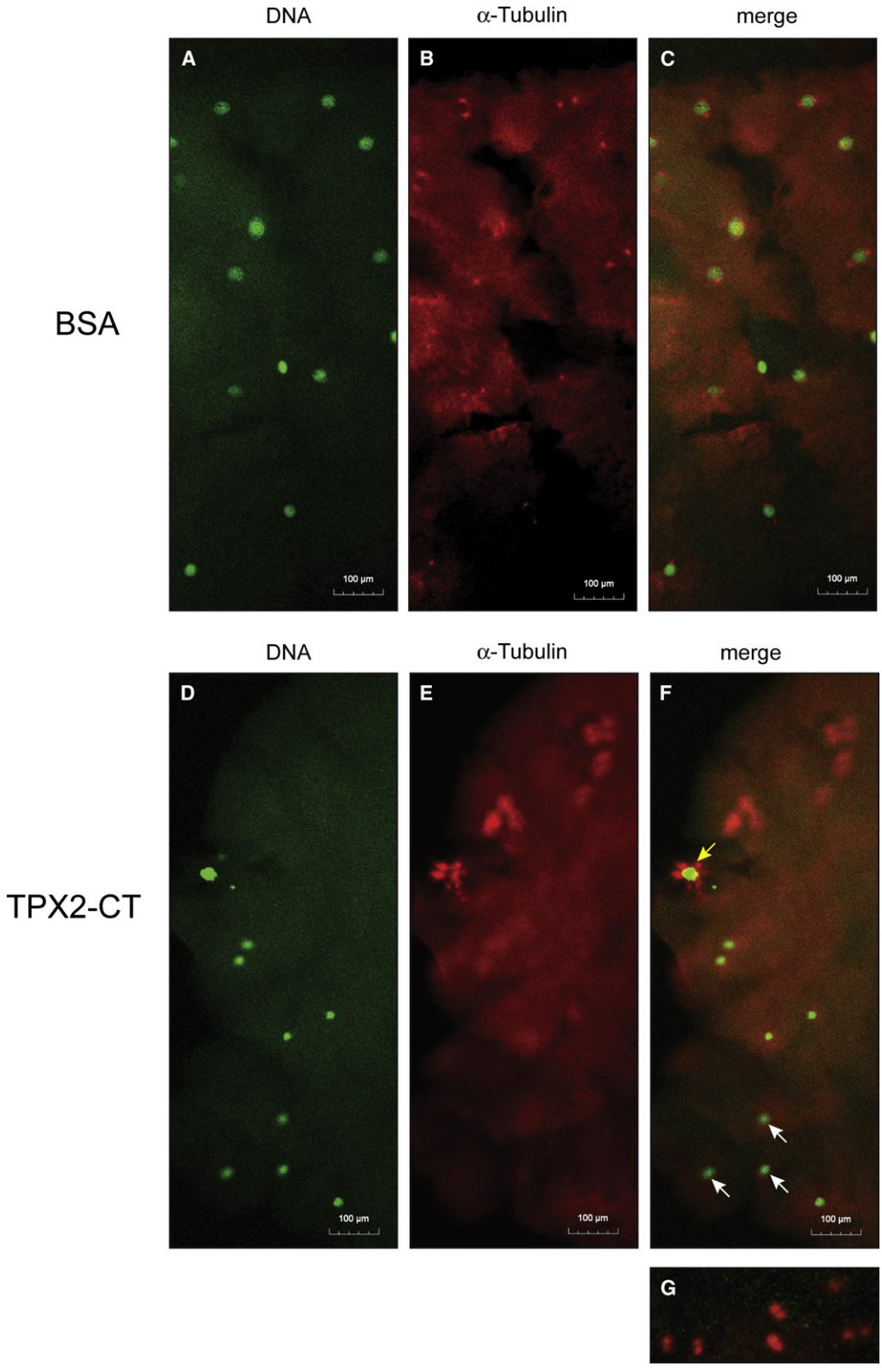


Figure S3. Injection of TPX2-CT Disrupts Spindles

CLSM analysis of embryos injected with TPX2-CT as described in Figure 1B. *Xenopus* embryos injected with TPX2-CT or BSA (control) were fixed ~3 hr after fertilization and stained for DNA (Sytox Green) and α-tubulin (red). Images were taken with the 10× objective. Control embryos were injected with BSA (A–C). TPX2-CT was injected to a final concentration of 0.5 μM.

(D–F). White arrows mark free nuclei, and the yellow arrow marks the nucleus associated with multiple spindle poles. A subset of chromatin free, pairwise-arranged centrosomes/spindle poles in a TPX2-CT-injected embryo is depicted in (G).

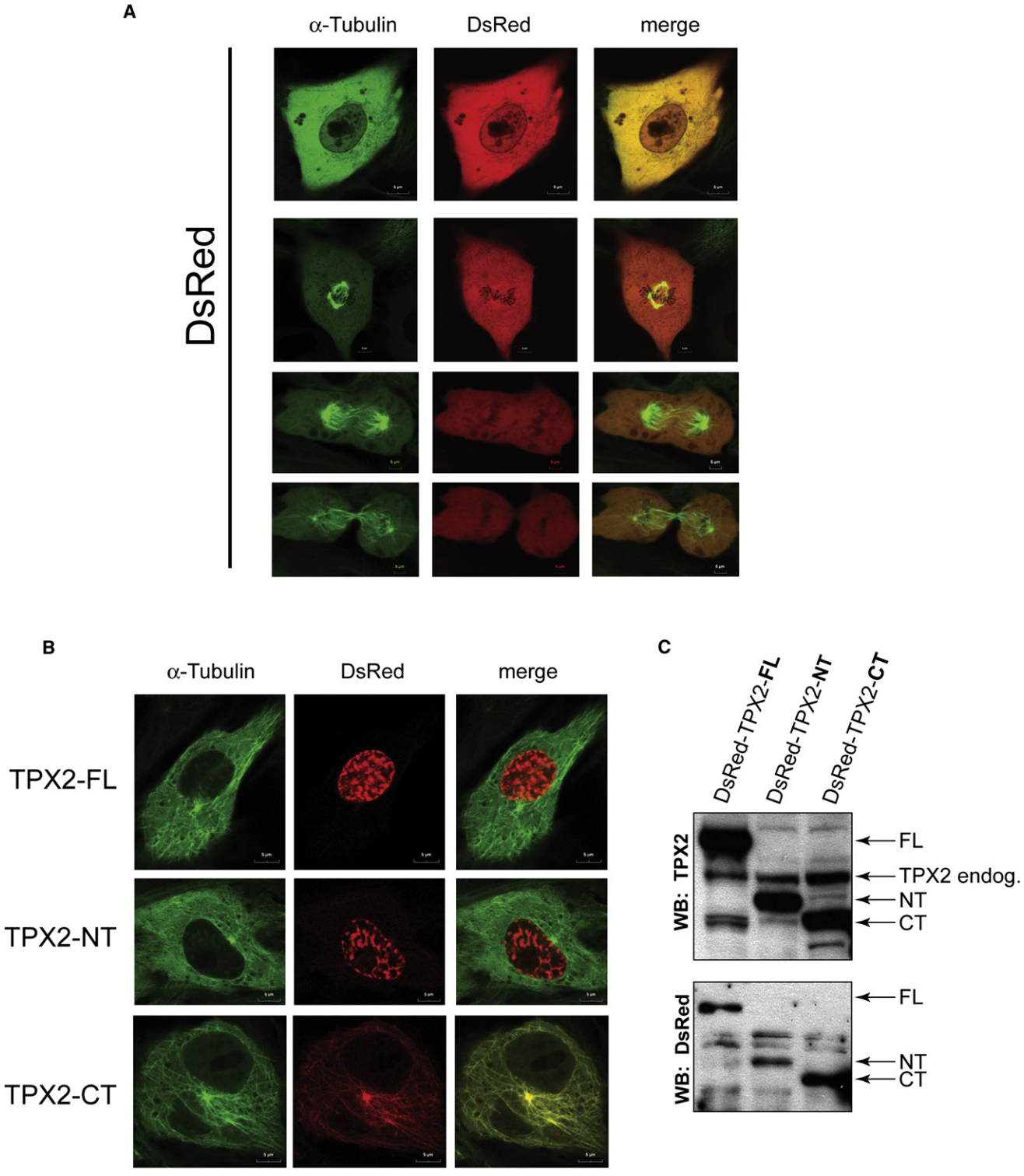
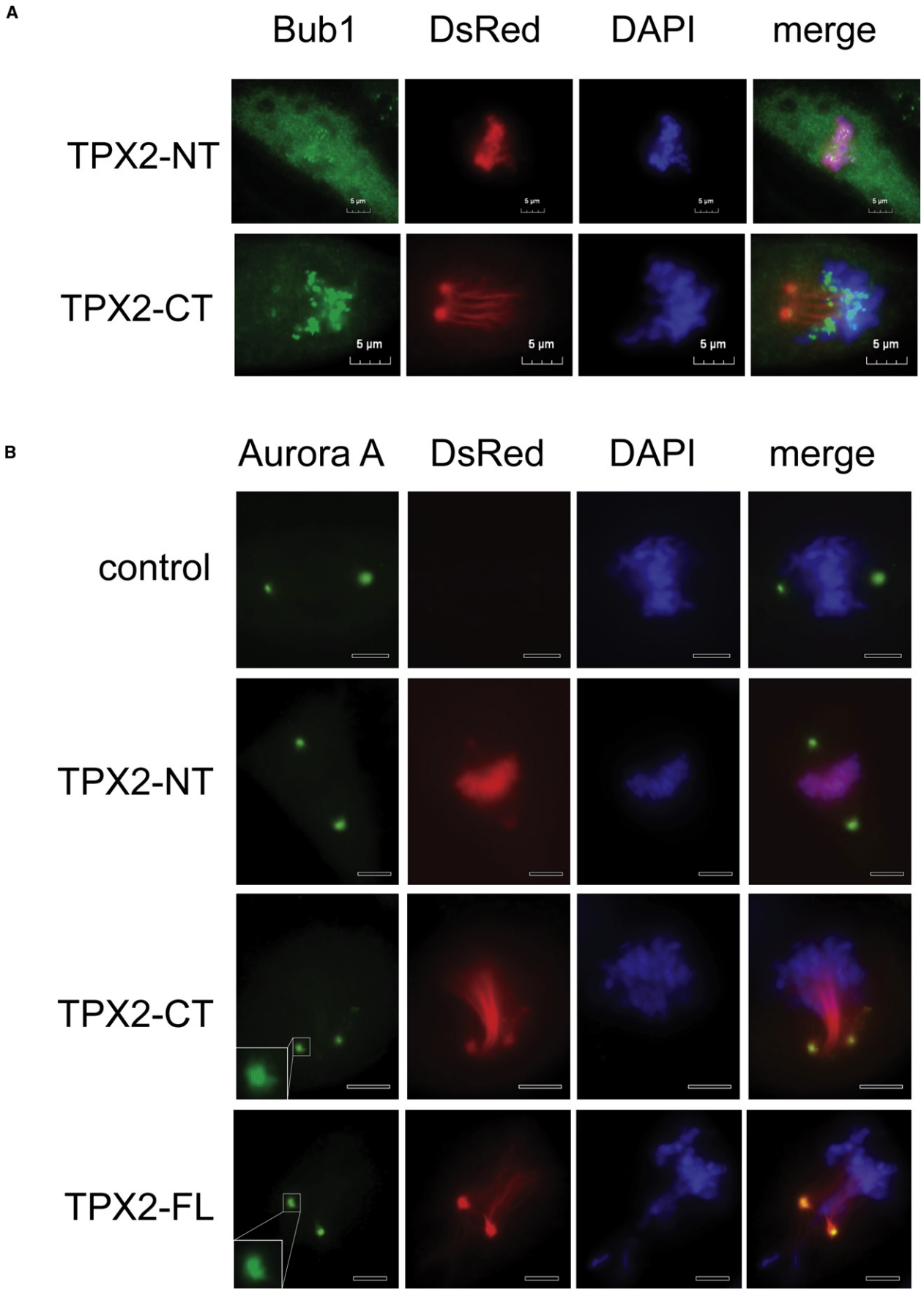


Figure S4. Localization of DsRed and DsRed-TPX2 Proteins in Living *Xenopus* S3 Cells

(A) CLSM analysis of GFP-α-tubulin (green) expressing X S3 cells, expressing DsRed protein (red) from the parental vector in different stages of mitosis. top panel, interphase; second panel, prometaphase; third panel, anaphase; lower panel, telophase.

(B) Subcellular localization of DsRed-tagged TPX2-proteins in interphase. DsRed-TPX2 (top panel), DsRed-TPX2-NT (middle panel) and DsRed-TPX2-CT (lower panel). X S3 cells were seeded onto glass-bottom wells and subjected to CLSM as described in the Supplemental Experimental Procedures.

(C) Western blot analysis of TPX2 expression levels. X S3 cells transfected with the indicated DsRed-TPX2 proteins were subjected to western blot analysis utilizing antibodies against TPX2 (top panel) and DsRed (bottom panel).


A
Bub1
DsRed
DAPI
merge
TPX2-NT
TPX2-CT
5 μm
B
Aurora A
DsRed
DAPI
merge
control
TPX2-NT
TPX2-CT
TPX2-FL

We next investigated whether TPX2-CT interferes with exit from mitosis at some point after securin degradation. For this purpose we utilized cytostatic factor (CSF)-arrested egg extracts that were released into interphase by addition of 0.5 mM $CaCl_2$. Because both Cdc27 dephosphorylation and cyclin B2 degradation occur in anaphase [S1], these proteins were monitored by western blot analysis for exit from mitosis. Whereas extracts incubated with Emi1-CT exhibited no downshift of Cdc27 nor any cyclin B2 degradation, extracts containing TPX2-CT or TPX2-NT showed an increase in Cdc27 mobility after 10 min, which was even more pronounced after 20 min (Figure S2B). Additionally, cyclin B2 was rapidly degraded in all these extracts consistent with mitotic progression into anaphase. We therefore conclude that TPX2-CT does not inhibit exit from mitosis, and thus these embryos are not arrested in mitosis.

TPX2-CT-Injected Embryos Biochemically Undergo Multiple Cell Cycles

Because TPX2-CT does not block exit from mitosis, we investigated whether TPX2-CT-injected embryos arrest in the subsequent S phase. Purified TPX2 proteins were injected into blastomeres together with [α-^{32}P]dCTP, and the synthesis of genomic DNA was monitored by agarose gel electrophoresis and autoradiography. Aphidicolin (APD), an inhibitor of DNA-polymerase α, also was coinjected as a positive control for efficient inhibition of DNA replication. Embryos injected with BSA alone incorporated radiolabel into DNA in a time dependent manner (Figure S2C). However, in embryos injected with BSA and APD, the incorporation of dCTP was barely detectable, demonstrating that APD efficiently inhibited DNA synthesis. Similarly, embryos injected with Emi1-CT failed to newly synthesize DNA due to mitotic arrest. By contrast, DNA synthesis continued unperturbed in embryos injected with either TPX2-NT or TPX-CT, as compared to embryos injected with BSA alone. Thus, TPX2-CT does not inhibit DNA synthesis.

Based on our observations, TPX2-CT-injected embryos could be arrested in S phase, performing multiple rounds of DNA replication (endoreplication), or these embryos may not be cell cycle arrested but, instead, are biochemically undergoing multiple cell cycles with cleavage furrow ingression defects and a subsequent failure in cytokinesis. In order to distinguish these possibilities, we analyzed embryos for their ability to reenter mitosis after the preceding interphase. Western blot analyses using antibodies directed against pTyr-15 in Cdc2 show that TPX2-CT-injected embryos progress through mitosis, and inhibitory phosphorylation of Tyr-15 of Cdc2 reappears (Figure S2D, middle). Similarly, cyclin B2 levels increase (Figure S2D, bottom), and Cdc27 shifts back to its slower migrating form, indicating it again becomes rephosphorylated in mitosis (Figure S2D, top). Similar phenotypes were observed with embryos injected with TPX2-NT, whereas embryos injected with Emi1-CT were arrested in mitosis as demonstrated by persistent Cdc27 phosphorylation (Figure S2D, top), no reappearance of inhibitory Tyr-15 phosphorylation (Figure S2D, middle) and high levels of cyclin B2 (Figure S2D, bottom). Monitoring histone H1 kinase activity in these embryos revealed that TPX2-CT-injected embryos undergo a second peak of histone H1 kinase activity 115 min after fertilization (Figure S2E). Taken together, these data show that TPX2-CT-injected embryos are not arrested in S phase but, rather, enter the subsequent mitosis and therefore are undergoing multiple cell cycles without cleavage furrow ingression. These results suggest that injection of TPX2-CT does not lead to a cell cycle arrest in these blastomeres but, rather, to a failure in cytokinesis.

Supplemental Experimental Procedures

Constructs, Protein Purification, and Pull-Down Assay

Cloning of *Xenopus laevis* TPX2 was described previously [S2]. For bacterial protein expression, TPX2-deletion constructs were generated by PCR and inserted into pET30 ek/LIC vector (Novagen) by LIC-cloning. Recombinant hexahistidine-tagged *Xenopus laevis* TPX2 was expressed in *Escherichia coli* BL 21 (DE3) by induction for 16–18 hr with 100 μM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and purified from the soluble bacterial fraction on Talon beads (Clontech). Proteins were dialyzed against TPX2-dialysis buffer (50 mM Tris [pH 7.4], 300 mM NaCl, 1 mM $MgCl_2$, 0.01% Brij 35, and 0.1% β-Mercaptoethanol) and stored in aliquots at -80°C. Emi1-CT protein was kind gift from Dr. Bryn Grimison (UCHSC). GST-Eg5-HS was described previously [S3, S4]. For pull-down assay, GST-proteins were expressed in BL21 (DE3), coupled to MagneGST beads (Promega), washed three times in Lysis buffer (250 mM NaCl, 50 mM Tris HCl [pH 8.0], 1% Triton X-100, and 10% Glycerol) followed by two times washing in TIF buffer (150 mM NaCl, 20 mM Tris HCl [pH 8.0], 1 mM $MgCl_2$, 0.1% NP-40, and 10% Glycerol). Subsequently, radiolabeled proteins generated by the TNT-Quick System (Promega) or 6His-TPX2 proteins purified from bacteria were added for 4 hr, followed by three times washing in TIF buffer and SDS-PAGE. For eukaryotic protein expression, TPX2-cDNAs were amplified by PCR and cloned into pDsRed2-C1 vector (Clontech) using EcoRI and BamHI restriction sites.

Immunization Procedures and Antibody Production

Resin containing purified His_6-TPX2 was used to immunize rabbits. Immune sera directed against TPX2 were affinity purified on an Affi-Gel 10 resin (Bio-rad) covalently crosslinked to TPX2. Monoclonal antibody against α-tubulin and polyclonal rabbit anti γ-tubulin antibodies were from Sigma-Aldrich, monoclonal antibody for Cdc27 was from BD Biosciences, and polyclonal anti pY15-Cdc2 was from Cell Signaling. Monoclonal β-actin antibody, monoclonal His antibody, and polyclonal GST antibodies were from Sigma-Aldrich and monoclonal DsRed antibody was from Clontech. Sheep anti-cyclin B2 antibody was prepared as described previously [S5], as were rabbit anti-Bub1 antibodies [S6]. Rabbit anti-Aurora B antibodies were a kind gift from Dr. Johné Liu (Ottawa Health Research Institute), and monoclonal anti-*Xenopus* Aurora A antibody has been described previously [S7].

Xenopus Embryos and CSF Extracts

X. laevis eggs were fertilized in vitro as described previously [S8]. For overexpression analyses, one blastomere of a two-cell embryo was injected with bacterially expressed protein to a final intracellular concentration of 0.5 μM, and cell division was monitored with a M7A Stereo Zoom Microscope (Wild, Heerbrugg). For rescue experiments, similar amounts of proteins were incubated together for ~1 hr in TIF buffer on ice before injection. Embryos used for biochemical analysis were injected in both blastomeres with 40 nl solution containing either bacterially expressed proteins alone, or together with 20 nCi of ^{35}S securin (10 nl) or 100 nCi of [α-^{32}P]dCTP (800 Ci/mmol) (10 nl). Embryos used for confocal microscopy were injected in both blastomeres. CSF extracts were prepared from unfertilized eggs as described [S9]. Western blotting and histone H1 kinase assays were performed as described previously [S10].

Figure S5. Bub1 and Aurora A Localization in TPX2-CT-Expressing *Xenopus* S3 Cells

(A) Analysis of the checkpoint protein Bub1 in X S3 cells expressing TPX2-proteins. Wild-type X S3 cells were seeded onto slide flasks, transfected, and methanol-fixed 8 hr after release from the second thymidine block. Bub1 was visualized by indirect immunofluorescence (green). DsRed-TPX2-NT and -CT were detected directly via the DsRed-tag (red). DAPI staining was used to visualize DNA (blue). Bar, 5 μm.

(B) Localization of Aurora A in X S3 cells expressing TPX2-proteins. Cells expressing the indicated proteins were treated as in (A), except Aurora A (green) was visualized by indirect immunofluorescence. Insets, enlarged Aurora A staining of spindle poles. Bar, 5 μm.

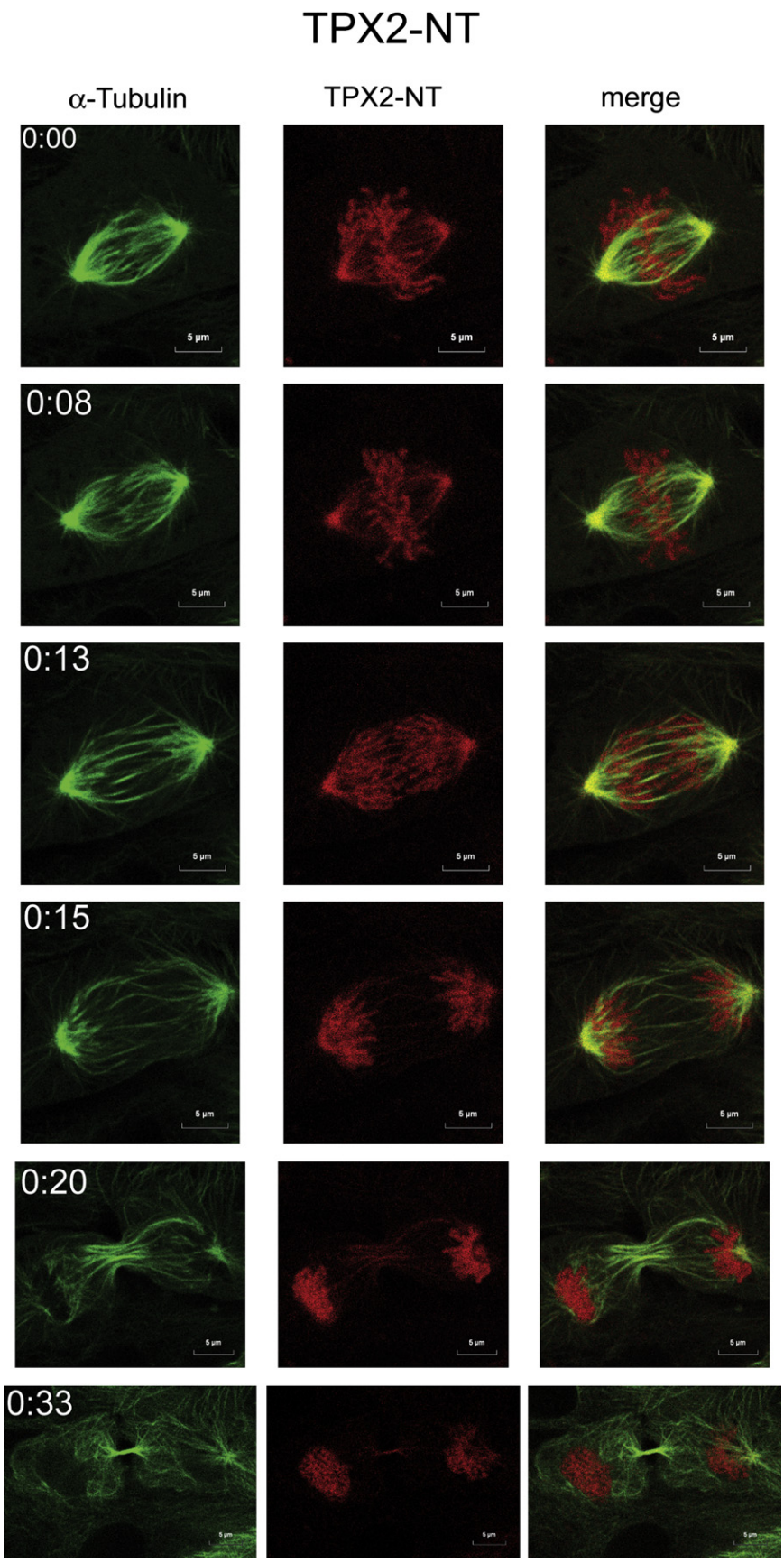


Figure S6. TPX2-NT Does Not Affect Mitosis

Live-cell imaging using CLSM of X S3 cells stably expressing GFP-α-tubulin and transiently transfected with DsRed-TPX2-NT, as described in Figure 3. Time (hr:min) after imaging began is indicated in the left panels.

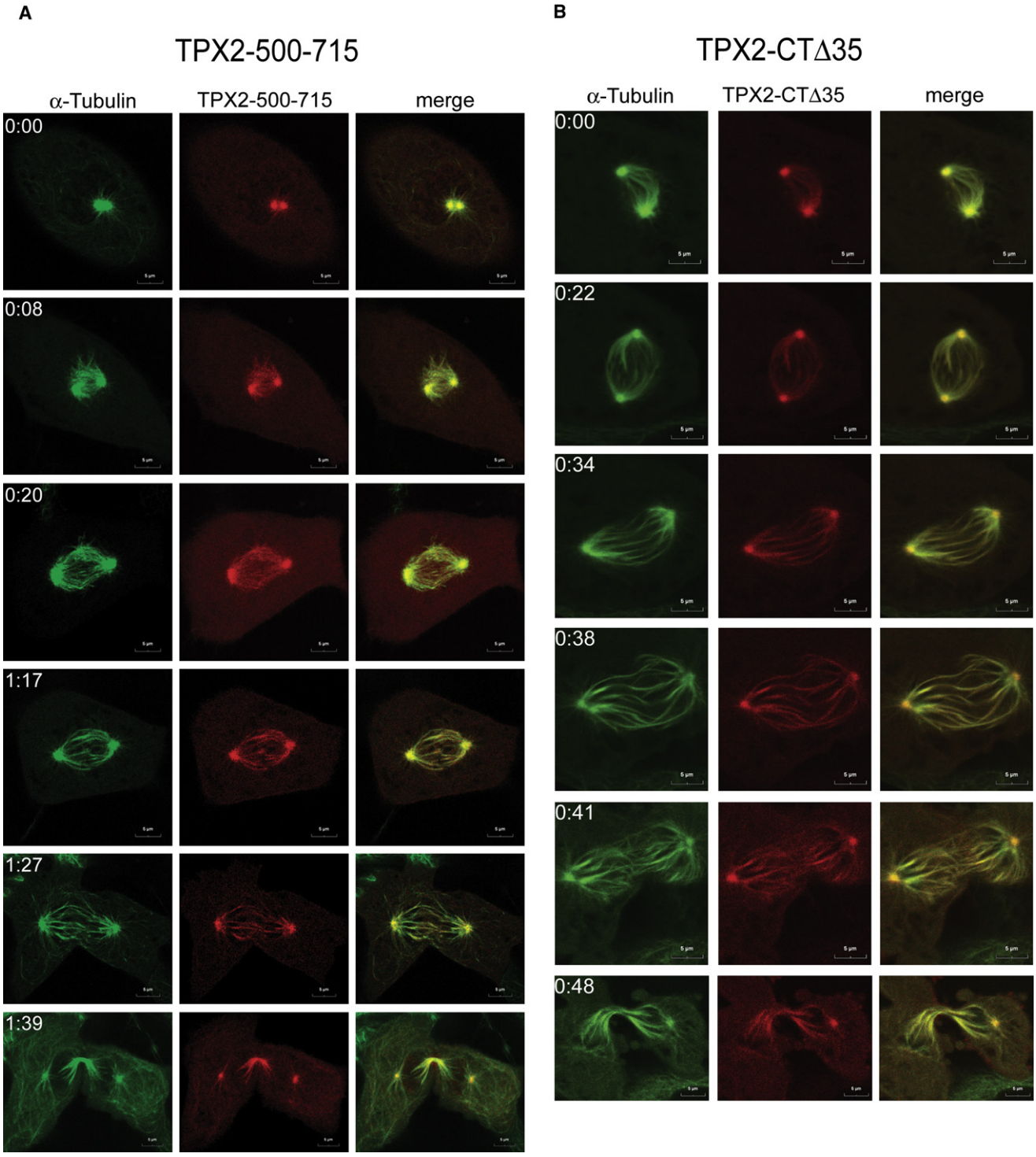


Figure S7. TPX2 (500–715), but Not TPX2-CTΔ35, Causes a Prolonged Metaphase

The same experiment as described in Figure S6 was performed with the indicated TPX2 constructs.
(A) DsRed-TPX2 (500–715) in an X S3 cell with extended metaphase (see hr:min in panels 3 and 4) throughout mitosis.
(B) DsRed-TPX2-CTΔ35 in an X S3 cell throughout mitosis.

Cell Culture

Xenopus S3 wild-type and GFP-α-tubulin-expressing cells were a kind gift from Dr. Gary Gorbsky (Oklahoma Medical Research Foundation) and have been described previously [S11]. *Xenopus* S3 cells were grown in Leibowitz's L-15 medium containing 15% FBS, 15% H_2O, and 2 mM L-glutamine at 25°C without CO_2. S3 cells stably expressing GFP-α-tubulin were cultured in selective medium containing 500 μg/ml G418. In general, cells were transfected immediately after trypsinization using Lipofectamine LTX in combination with PLUS reagent (Invitrogen) according to the manufacturer's instructions. Subsequently, cells underwent double thymidine treatment (2 mM thymidine for 14–16 hr, 8 hr release followed by a second thymidine treatment) to synchronize cells at the G_1/S boundary. Monitoring

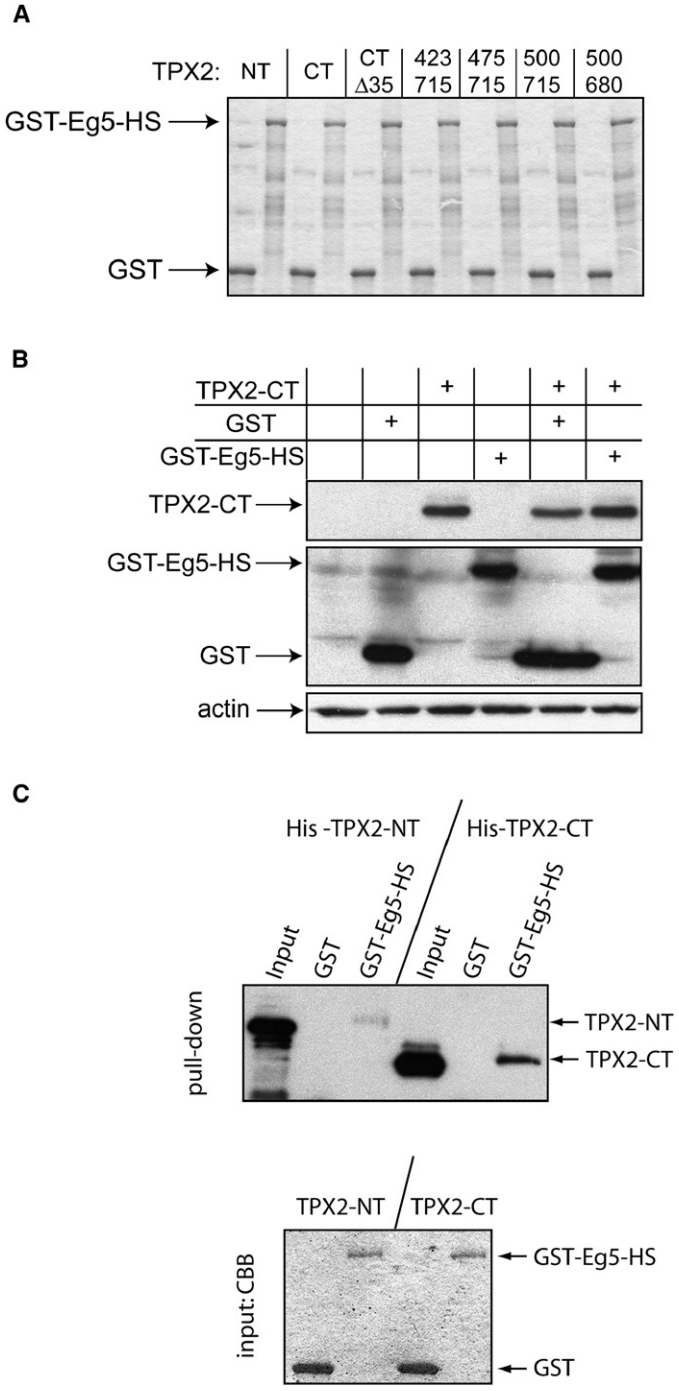


Figure S8. Interaction of TPX2-CT Domains with Eg5

(A) Pull-down input control. Coomassie staining of one-fourth of the pull-down reactions shown in Figure 4A reveals similar input amounts of GST and GST-Eg5-HS proteins.

(B) Western blot control of injected proteins. Western blot analysis of embryos (depicted in Figure 4B) shows that similar amounts of GST and GST-Eg5-HS were coinjected with 6his-TPX2-CT into embryos. For each injection, ten embryos were crushed and the amount corresponding to one embryo was subjected to SDS-PAGE followed by western blot analysis. The amount of injected His-TPX2-CT protein was detected by using anti-His antibodies (top panel). Similar amounts of GST and GST-Eg5-HS were injected as judged by anti-GST western blot analysis (middle panel). Western blotting of actin confirmed equal loading of the gel (bottom panel).

(C) Investigation of Eg5 and TPX2 interaction. GST-Eg5-HS pull-down analysis of His-tagged TPX2 proteins purified from bacteria, as described in the Supplemental Experimental Procedures. Top: Western blot analysis using anti His-antibody to detect GST-Eg5-HS-interacting TPX2 proteins. Input is 20%. Bottom: one-fourth of the reaction was subjected to SDS-PAGE and Coomassie staining to depict the amounts of GST-proteins used in the reaction.

of cells started 5 hr after release from the second thymidine block. For live-cell imaging, cells were seeded onto 0.17 mm glass-bottom wells (WillCo Wells, Netherlands). Live-cell imaging was performed at room temperature in L-15 medium.

For analysis by immunofluorescence, cells were seeded onto slide flasks (Nunc). Cells were fixed with ice-cold methanol and nonspecific epitopes were blocked with PBS containing 1% BSA, followed by incubation with primary antibody and then secondary antibodies conjugated with Alexa Fluorochromes AF488 and AF594 (Invitrogen). Slides were mounted in ProLong Gold antifade reagent (Invitrogen).

Microscopy and Confocal Laserscan Microscopy

Microscopy was performed by using a Nikon Eclipse TE 300, PCM 2000 inverted microscope at room temperature. Objective lenses were from Nikon and included the following: 10× air Plan Fluor objective, NA 0.30; 40× air Plan Fluor objective, NA 0.75; 100× oil Plan Apo objective, NA 1.40. Fluorochromes were from Invitrogen and included AF488 (green), AF555 (red) and AF633 (far red). Confocal methods were performed essentially as described previously [S12]. For confocal laser scanning microscopy (CLSM) of embryos, DNA was stained with Sytox Green and for *Xenopus* S3 cells, DNA was stained with DAPI. Images for non-confocal microscopy were taken with an air cooled charge-coupled device (CCD) camera, (SenSys, Photometrics) attached to a 0.76× coupler (Diagnostic Instruments) and for the dissecting microscope pictures were taken with a Nikon Coolpix 990 camera equipped with a Coolpix MDC Lens (0.82-0.29). For microscopic analysis, the acquisition software Simple PCI (Compix) was used. In some cases (Figure 1B and Figure S6A) selected images taken from a Z stack were merged by using the montage function to visualize several planes of the specimen. No other software for image processing was used.

Supplemental References

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