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UXT Is a Novel Centrosomal Protein Essential for Cell Viability[D]

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Ubiquitously expressed transcript (UXT) is a prefoldinlike protein that has been suggested to be involved in human tumorigenesis. Here, we have found that UXT is overexpressed in a number of human tumor tissues but not in the matching normal tissues. We demonstrate that UXT is located in human centrosomes and is associated with γ-tubulin. In addition, overexpression of UXT disrupts centrosome structure. Furthermore, abrogation of UXT protein expression by small interfering RNA knockdown leads to cell death. Together, our findings suggest that UXT is a component of centrosome and is essential for cell viability. We propose that UXT may facilitate transformation by corrupting regulated centrosome functions.

INTRODUCTION

The centrosome is the major microtubule-organizing center in animal cells. It consists of two centrioles associated with an amorphous, electron-dense pericentriolar material referred to as the centrosome matrix. A multiprotein complex known as the γ-tubulin ring complex disposed within the centrosome matrix is required for both microtubule nucleation at the centrosome (Zheng *et al*., 1995) and organization of centrioles (Ruiz *et al*., 1999).

The normal G_1 stage cell contains a single centrosome, which is duplicated as DNA replicates during the S phase. The precisely duplicated centrosomes separate during the G_2 phase, each localized to the spindle pole during the M phase of the cell cycle. Abnormalities of composition of the centrosomes or aberrant numbers of centrosomes have been detected in a variety of tumors (Lingle *et al*., 2002; Jiang *et al*., 2003; Yamamoto *et al*., 2004). The notion that abnormal centrosomes contribute to the progressive malignant phenotype by causing incorrect chromosome segregation and aneuploidy is gaining acceptance (Nigg, 2002).

Molecular chaperones play critical roles in many fundamental cellular processes (Hartl and Hayer-Hartl, 2002), including centrosome behavior. For example, Hsp90 is a core centrosomal component and is essential for certain centrosome functions (Lange *et al*., 2000). In addition, the tailless complex polypeptide 1 (TCP-1) family of chaperones is also localized to the centrosome (Brown *et al*., 1996) and facilitates γ-tubulin maturation (Melki *et al*., 1993). Delivery of newly synthesized γ-tubulin to TCP-1 requires a member of the prefoldin (Gim-complex, genes involved in microtubule biogenesis) family of chaperones (Vainberg *et al*., 1998). Notably, the characterized prefoldin is a hexameric molecular chaperone complex built from two related classes of subunits (Siegert *et al*., 2000) and is proposed to play a general role in de novo protein folding (Lundin *et al*., 2004). Loss of prefoldin impairs the function of the centrosome (Le Bot *et al*., 2003) and can affect cell viability (Maeda *et al*., 2001).

Cdc14A is a human homologue of the multifunctional protein phosphatase Cdc14 that can antagonize cyclin-dependent kinase activities (Simons *et al*., 2004). It is localized to the centrosome and seems to be important for centrosome separation (Simons *et al*., 2004). In this study, we have discovered that UXT is a binding partner of Cdc14A through yeast two-hybrid screens.

Because of its abundance in tumor samples, UXT has been thought to be involved in tumorigenesis. For example, a majority of the expressed sequence tag (EST) clones corresponding to UXT are derived from a variety of tumor cell lines, and UXT seems to be overexpressed in these tumor tissues (Schroer *et al*., 1999). Here, we demonstrate that UXT is a novel component of the centrosome and is associated with γ-tubulin. In addition, overexpression of UXT causes loss of pericentriolar material. Furthermore, knockdown of the expression of UXT promotes p53-independent cell death. We propose that UXT may contribute to tumorigenesis by corrupting centrosome activity.

MATERIALS AND METHODS

Reagents and Antibodies

The rabbit anti-γ-tubulin antibody, the monoclonal anti-γ-tubulin, anti-FLAG, and anti-green fluorescent protein (GFP) antibodies were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Culture and Treatments

The human osteoblast sarcoma U2 (U2OS) cell line and the human embryonic kidney 293T (HEK293T) cell line were obtained from the American Type Culture Collection (Manassas, VA). These cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA) and 1% antibiotics at 37°C with 5% CO_2. All transfections were carried out using FuGENE 6 (Roche Diagnostics, Indianapolis, IN) following the manufacturer's instructions.

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Yeast Two-Hybrid Screening

The Matchmaker GAL4 two-hybrid system 3 (BD Biosciences Clontech, Palo Alto, CA) was used to perform yeast two-hybrid screening. The Gal4-fused full-length Cdc14A expressed from pGBTK7 plasmid was used as bait. Transformants (2×10^6) from a HeLa cDNA library (pGAD-GH plasmid; BD Biosciences Clontech) were screened in the yeast strain AH109 (BD Biosciences Clontech) and 11 colonies were identified as positive clones. Among these 11 positive colonies, seven were revealed to encode UXT.

Vectors

To generate pcDNA3-FLAG:UXT, full-length UXT was subcloned from the clone isolated from yeast two-hybrid screening by digestion with *Eco*RI and *Xba*I and ligation to the pcDNA3 vector (Invitrogen) that contains two FLAG tag sequences between the *Hind*III/*Eco*RI sites. The pEGFP:UXT was constructed by inserting UXT at the *Eco*RI/*Xho* I site into pEFGPC1 (BD Biosciences Clontech). Enhanced green fluorescent protein (EGFP):Cdc14A was constructed by inserting Cdc14A amplified by PCR from a plasmid containing full-length Cdc14A (a gift from Dr. H. Yu, University of Texas Southwestern Medical Center, Dallas, TX) into pEGFPC1 at the *Eco*RI/*Xho*I sites. The γ-tubulin/green fluorescence protein (TGFP)-expressing plasmid was kindly provided by Dr. Alexey Khodjakov (Wadsworth Center, New York State Department of Health, Albany, NY). All the plasmid sequences were verified by automated DNA sequencing.

Coimmunoprecipitation

The HEK293T cells (2×10^6) were transfected with various combinations of expression plasmids (4 μg each) by using FuGENE 6. After 48 h, cells were harvested, washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in 800 μl of lysis buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, and 0.5% Nonidet P-40) containing 1 mM sodium orthovanadate, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1% protease inhibitor cocktail (Sigma-Aldrich) for 15 min on ice. After lysis, cells were centrifuged at $13{,}000 \times g$ for 10 min at 4°C, and the protein content was determined using the bicinchoninic acid assay (Pierce Chemical, Rockford, IL). Total cell lysate (500 μg) was precleared with protein G agarose beads and mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for 1 h. FLAG:UXT, EGFP-UXT, FLAG-Cdc14A, or EGFP-Cdc14A, γ-tubulin-GFP was then immunoprecipitated using the anti-GFP or anti-FLAG monoclonal antibody (mAb) combined with the protein G agarose beads. The immune complex was then washed four times with the washing buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Nonidet P-40) and subjected to SDS-PAGE. The proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA), and the blots were probed with different antibodies. The secondary antibody coupled with horseradish peroxidase (Santa Cruz Biotechnology) was detected by enhanced chemiluminescence reagents (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom).

Stable Cell Line

The human U2OS osteoblast sarcoma cell line was transfected using the FuGENE 6 reagent. The G418-resistant clones were screened for expression of GFP fusion proteins using a Nikon fluorescent microscope equipped with a xenon lamp power supply and a GFP filter set. Fluorescent positive clones were further analyzed by Western blot using the anti-GFP mAb or the mouse anti-UXT antibody.

Indirect Immunofluorescence

Cells were cultured, fixed either in methanol or in a paraformaldehyde/Triton X-100 solution, and immunostained following a standard protocol. Controls using equivalent amounts of preimmune IgG or without primary antibody were included in each experiment. The samples were stained with 4,6-diamino-2-phenylindole (DAPI), mounted on slides using Vectashield (Vector Laboratories, Burlingame, CA), and imaged by using Laser Scanning Microscope 410 (Carl Zeiss, Thornwood, NY).

Electron Microscope

U2OS cells transfected with EGFP or EGFP:UXT were washed with PBS and fixed with 2.5% glutaraldehyde for 60 min at room temperature. After post-fixation with 1% OsO_4 for 60 min, the samples were dehydrated through an ethanol series and embedded in Epon-Araldite plastic. Thin sections were cut on a LKB Nova ultramicrotome and stained with uranyl acetate and lead citrate.

Generation and Transfection of Small Interfering RNA (siRNA)

We used the following target sequences for UXT siRNA: ^{434}UAC AAG GCC UGC AGA AUU U^{452} and ^{362}GCA ACA GCC UCA CCA AGG A^{580}. We obtained similar results using these two oligonucleotides. The data presented here were obtained using the latter oligonucleotide. U2OS cells were transfected with siRNAs in six-well dishes by using 3 μl of Oligofectamine reagent (Invitrogen) and diluted in serum- and antibiotic-free Opti-MEM in a final volume of 0.2 ml and containing the following amounts of siRNA: 5 μl of 20 μM solution per transfection for the siRNA. The plating medium volume was 1300 μl per transfection. After 4 h of transfection in serum-free Opti-MEM, 500 μl of Opti-MEM plus 30% FBS were added to each transfection. The final siRNA concentration was 50 nM. We used the nonspecific control siRNA duplexes (Dharmacon, Chicago, IL) as the control.

Flow Cytometry and Cell Cycle Analysis

U2OS cells were transfected with siRNA duplexes. At indicated times, we processed floating and adherent cells for flow cytometry analysis. Briefly, cells were suspended in NP-40 solution (0.1% sodium citrate, 0.0564% NaCl, 0.03% NP-40) with 200 μg ml-1 RNase and stained with 20 μg ml-1 propidium iodide. Cells were analyzed for DNA content. We evaluated dead cells as the percentage of subG1 cell population calculated with MultiCycle software.

RESULTS

UXT Interacts with the Centrosomal Protein Phosphatase Cdc14A

Using a yeast two-hybrid protein interaction trap, we sought to identify proteins that were able to interact with Cdc14A. Yeast transformants (2×10^6) were screened in a human HeLa cell cDNA library. DNA sequencing and database searching revealed that seven of 11 positive clones encoded UXT. The interaction specificity between the Cdc14A and UXT was confirmed by the yeast two-hybrid assay. Only cotransformation of the Cdc14A bait and the UXT clone, but not control vectors, rendered yeast cells the ability to grow on the selective media (Figure S1).

To verify that the interaction between Cdc14A and UXT also occurs in mammalian cells, we cotransfected EGFP:Cdc14A and a FLAG-tagged UXT form into HEK293T cells and performed coimmunoprecipitation. As shown in Figure 1A, the 98-kDa EGFP:Cdc14A fusion protein was coprecipitated with the FLAG:UXT by the anti-FLAG antibody. Conversely, the FLAG:UXT was coprecipitated with EGFP:Cdc14A, but not GFP (Figure 1B). Examination of the UXT–Cdc14A interactions at the endogenous level by the same approach must await new reagents because available antibodies against UXT and hCdc14A are not effective in coimmunoprecipitation analysis.

UXT Is Localized to the Centrosome

To investigate the cellular localization of UXT, we generated an EGFP:UXT fusion construct and established a U2OS cell line with stably expression of the fusion protein. By immunofluorescence analysis, we found that EGFP:UXT formed one or two dots in each cell, which were colocalized with the centrosomal marker protein γ-tubulin (Figure 2A). Immunostaining of U2OS stably expressing EGFP:UXT with anti-α-tubulin antibodies indicated that EGFP:UXT localized to the spindle poles during mitosis (Figure 2B). In comparison, the EGFP protein alone is homogenously distributed in U2OS or HeLa cells (our unpublished data). To further eliminate the possibility that the centrosomal localization was caused by the EGFP tag, we created a FLAG-tagged UXT construct. Coimmunostaining of γ-tubulin and the FLAG epitope confirmed that the ectopic FLAG-tagged UXT was targeted to the centrosome (Figure 2C). Thus, we conclude that UXT is localized to the centrosome. Notably, nocodazole treatment of the cells has no effect on the centrosomal localization of GFP-tagged or FLAG-tagged UXT (our unpublished data), indicating that UXT is an integral component of the centrosome.

Identification of Structural Features Required for UXT Localization to the Centrosome

To determine the structural features involved in UXT localization, we generated a number of UXT mutants and exam-

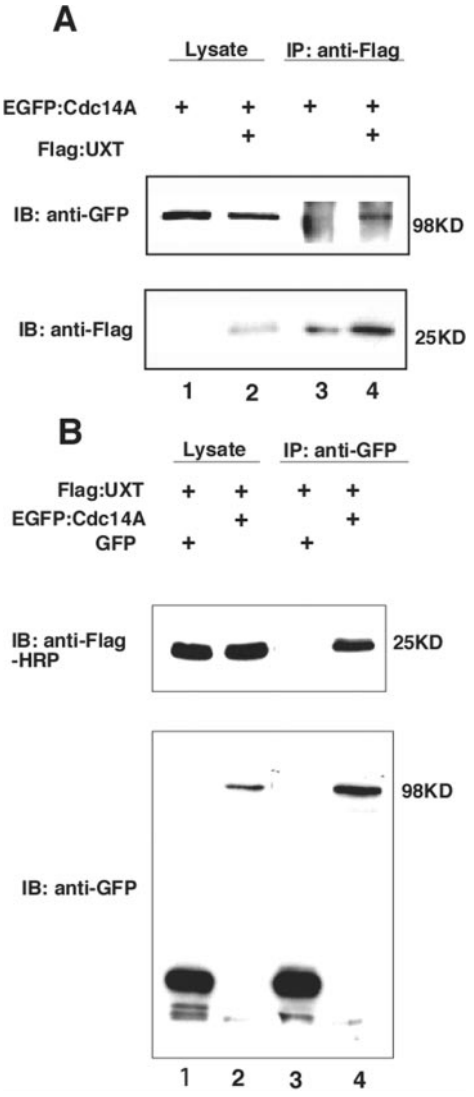


Figure 1. Coimmunoprecipitation analysis of the interaction between Cdc14A and UXT in HEK293T cells. (A) HEK293T cells were transfected with a combination of plasmids, including pEGFP:Cdc14A, pFLAG:UXT, and the control empty vector. The lysates were subject to immunoprecipitation using anti-FLAG antibody (M2), followed by Western blot using either an anti-GFP antibody (top) or the anti-FLAG antibody (bottom). Top, expression of EGFP:Cdc14A in the lysate and the presence of EGFP:Cdc14A in proteins coprecipitated with FLAG:UXT. Bottom, expression of FLAG:UXT in the cell lysate and in the immunocomplex precipitated by the anti-FLAG antibody. Note that the band corresponding to the FLAG:UXT protein overlaps with the mouse IgG light chain (lane 4). (B) HEK293T cells were transfected with a combination of plasmids, including pEGFP:Cdc14A, pFLAG:UXT, and the control EGFP vector. The lysates were subject to immunoprecipitation using anti-GFP antibody, followed by Western blot using either an anti-FLAG antibody (top) or the anti-GFP antibody (bottom). Top, expression of FLAG:UXT in the lysate and the presence of FLAG:UXT in proteins coprecipitated with EGFP:Cdc14A. Bottom, expression of EGFP:Cdc14A in the cell lysate and in the immunocomplex precipitated by the anti-GFP antibody.

ined their distribution in the cell. We found that UXT contains a short coiled-coil domain in the region between amino acid residues 49–62. We noted that UXT was structurally related to the prefoldin family proteins, which assist the biogenesis of actins and tubulins as well as other proteins (Salisbury, 2003). The interaction between prefoldins and their substrates depends on coiled-coil motifs. The organization of centrosomal pericentriolar material also involves multiple coiled-coil proteins (Walton and Sousa, 2004). We therefore created two point mutations (L50P and L59P) in this region that presumably disrupted the coiled-coil domain. Indeed, we found that mutagenesis of the two leucine residues caused the dislocation of UXT from the centrosome (Figure S2).

As shown in Figure 3A, UXT contains five α-helixes. We created five deletion mutants of UXT based on the predicted secondary structure and determined their localization in U2OS cells. The results of the localization study are summarized in Figure 3B. The confocal microscopy images of these mutants were shown as supplemental data (Figure S1). We found that the three centrosomal localized mutants (GFP:UXT1-148, GFP:UXT9-157, and GFP:UXT9-148) encompass all the five α-helixes. Deletion of the N-terminal (GFP:UXT25-157) or C-terminal (GFP:UXT1-129) α-helix abolished centrosomal localization (Figure S2). These data suggest that although the distal amino acid segments at each end of the UXT polypeptide are not essential for centrosomal localization, both the N-terminal and C-terminal α-helix as well as the coiled-coil region are required for targeting UXT to the centrosome.

Generation and Characterization of UXT Antibodies

To determine the localization of endogenous UXT molecules and to assess the contribution of altered expression of UXT protein in cancer, we generated several monoclonal anti-UXT antibodies. Purified His-tagged UXT proteins were used to immunize mice. Three independent hybridoma cell lines were established. These antibodies, designated 1B2, 15A6, 6D3, were immunologically subtyped as IgA(1B2) and IgM(15A6, 6D3), respectively. In Western blot analysis, 1B2 can recognize His-tagged UXT (our unpublished data), FLAG-tagged UXT (Figure 4, lane 3), EGFP-tagged UXT (lane 2) as well as a single band in the lysates prepared from several human cell lines (Figure 4A, lane 1). The 1B2 antibody, however, was not suitable for immunostaining of fixed cells.

The antibodies 15A6 and 6D3 could readily detect the UXT protein in enzyme-linked immunosorbent assays and immunofluorescence studies, but they proved to be not effective for Western blotting. Interestingly, 15A6 only binds to the unreplicated centrosome (Figure 4B), whereas 6D3 detects an epitope on the centrosome only during the G_2/M stage (Figure 4C). Centrosomal staining signals were lost after adsorption of the two antibodies with purified UXT protein before immunostaining (our unpublished data), which thereby confirmed that the antibodies were specific for UXT. Collectively, these results suggest that the antibodies 15A6 and 6D3 bind to the native conformation of the UXT but not the denatured protein. We propose that the differential staining patterns by theses antibodies may reflect cell cycle-dependent conformational or dispositional changes of the UXT. It is possible that certain epitopes are obscured at a specific stage of the cell cycle as a result of either posttranslational modification or association with other proteins.

Overexpression of UXT in Some Human Tumors

It has been proposed that UXT may be involved in tumorigenesis because an EST database survey indicated UXT is abundantly expressed in a variety of tumor tissues (Schroer *et al*., 1999). There are no clear data to support such a

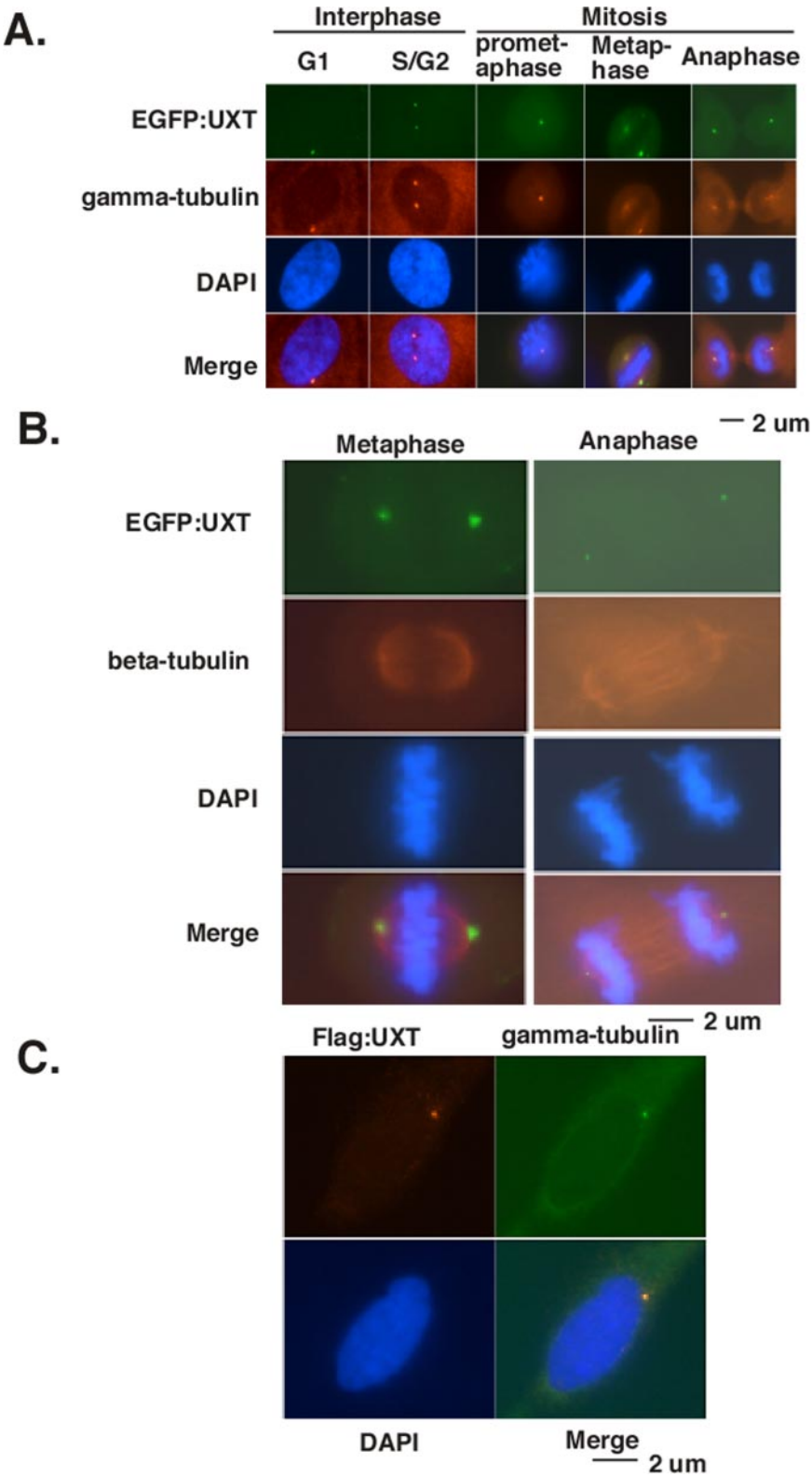
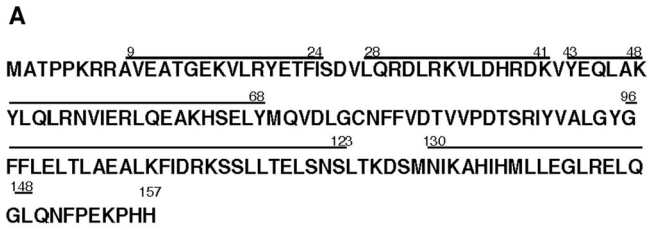


Figure 2. UXT is a novel centrosomal component. (A) Colocalization of UXT with the centrosome marker protein γ-tubulin. U2OS cells stably expressing EGFP:UXT (green) were labeled with anti-γ-tubulin (red). Images of cells in the G_1, S/G_2, prometaphase, metaphase, and anaphase are shown. (B) GFP-UXT is localized to the spindle poles during mitosis. U2OS cells stably expressing EGFP:UXT (green) were stained with an anti-α-tubulin (red) antibody. (C) The FLAG:UXT protein is localized to the centrosome. U2OS cells transfected with FLAG:UXT were immunostained with the mouse anti-FLAG antibody (red) and the rabbit anti-γ-tubulin antibody (green).

hypothesis. The DiscoverLight tissue lysate arrays (Pierce Chemical) provide a convenient method for protein expression screening. 38 tissue samples (corresponding to 19 pairs of human normal and tumor tissue lysates) were examined with the UXT-specific antibody 1B2. Our data indicate that UXT protein levels were elevated in several tumor tissues, including bladder, breast, ovary, and thyroid, but not in the matching normal tissues (Figure 5A). These results were verified by Western blot analyses using DiscoverLight tissue lysate sets (Figure 5B).



B

Construct	Centrosomal Localization
EGFP only	Diffuse
UXT1-157	Yes
UXT9-157	Yes
UXT1-148	Yes
UXT9-148	Yes
UXT1-129	No
UXT25-127	No
UXTL50P	No
UXTL59P	No

Figure 3. Subcellular localization of mutant UXT proteins. (A) Amino acid sequence of UXT: the predicted α-helixes were indicted with lines above the corresponding amino acids. (B) Summary of the localization patterns of the GFP-tagged UXT mutants.

UXT Is Associated with γ-Tubulin

We performed a yeast two-hybrid screen of a *Hela* cDNA library with the full-length UXT as bait and isolated the cDNA encoding amino acids from 322 to 451 of γ-tubulin. The specificity of the interaction between UXT and γ-tubulin was confirmed by the yeast two-hybrid assay (our unpublished data). To test whether UXT was also a physiological γ-tubulin binding partner, we performed coimmunoprecipitation assays. High-affinity and specific antibodies are not available for immunoprecipitation of endogenous UXT or γ-tubulin. We used the seminative coimmunoprecipitation approach. GFP-tagged γ-tubulin or FLAG-tagged UXT was introduced into HEK293T cells, respectively. The anti-FLAG antibody was used to immune precipitate FLAG-tagged UXT. As shown in Figure 6A, endogenous γ-tubulin was coimmunoprecipitated with FLAG-tagged UXT. Conversely, the anti-GFP antibody was used to immune precipitate GFP-tagged γ-tubulin. As shown in Figure 6B, endogenous UXT was coimmunoprecipitated with GFP-tagged γ-tubulin. Endogenous α-tubulin was not coimmunoprecipitated with UXT (our unpublished data), eliminating the possibility that γ-tubulin was coimmunoprecipitated with UXT because of its abundance in the cells.

Disruption of the Centrosome Structure by Overexpression of UXT

We documented that the EGFP:UXT is localized in the centrosome in the stably transfected U2OS cell line (Figure 2). To investigate the centrosomal functions of UXT, we transiently expressed EGFP:UXT in U2OS cells and assessed the changes of the centrosome by immunostaining with the anti-γ-tubulin antibodies. Intriguingly, 95% of U2OS cells with high EGFP:UXT expression, which is defined as formation of more than two aggregates in the cytosol, showed loss or dramatic reduction of γ-tubulin staining (Figure 7A). As a control, only 8% of EGFP-overexpressing cells showed reduced γ-tubulin staining (our unpublished data). Consistent with the centrosome activity in microtubule nucleation and stabilization, over expression of UXT also reduced the microtubule number and affected the distribution of the micro-

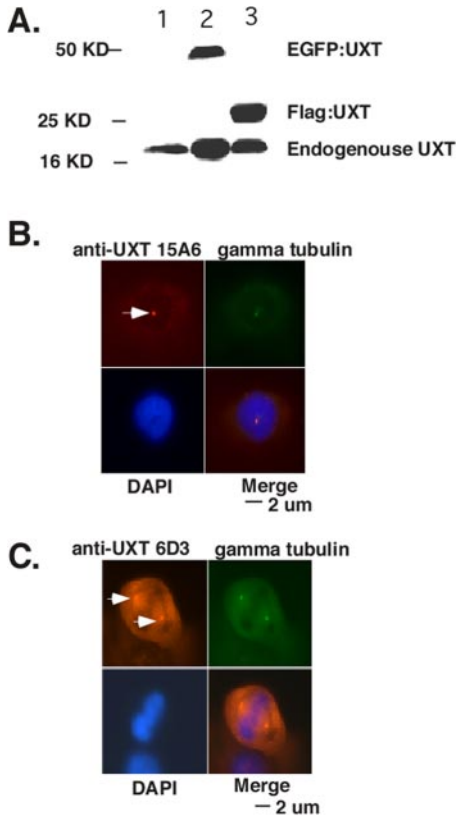


Figure 4. Characterization of the anti-UXT antibodies. (A) The anti-UXT antibody 1B2 can detect both the endogenous UXT and the UXT fusion proteins. Cell lysates from HEK293T cell (lane 1), HEK293T cells transfected with EGFP:UXT (lane 2), and HEK293T cells transfected with FLAG-tagged UXT (lane 3) were separated by 15% SDS-PAGE. The expression of UXT was examined with the anti-UXT antibody 1B2 by Western blot analysis. Note that the anti-UXT antibody 1B2 can detect the endogenous UXT protein (18KD), EGFP:UXT (50KD), and FLAG:UXT (25KD). (B and C) Immunofluorescence detection of the endogenous UXT proteins in U2OS cells using the anti-UXT antibodies. U2OS cells were fixed with methanol and stained with anti-γ-tubulin antibody (green) in combination with either the anti-UXT antibody 15A6 (B, in red) or the anti-UXT antibody 6D3 (C, in red). DNA was stained with DAPI (blue). The arrows indicate the centrosomes.

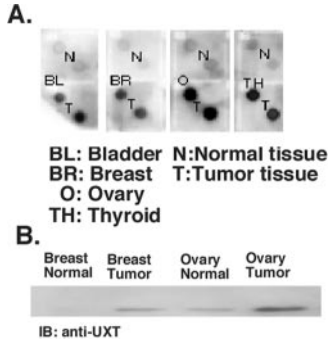


Figure 5. The UXT protein is overexpressed in several tumor tissues. (A) UXT is overexpressed in bladder (B), breast (BR), ovary (O), and thyroid (TH) tumors (T). The DiscoverLight human normal and tumor tissues lysate array (Pierce Chemical) is probed with the anti-UXT antibody 1B2. The lysates from the corresponding normal tissues (N) were used as control. (B) Detection of UXT protein levels in breast and ovarian tumors by Western blot. Lysates prepared from normal or tumor tissues (breast and ovary) were separated by 15% SDS-PAGE and probed with the anti-UXT antibody 1B2.

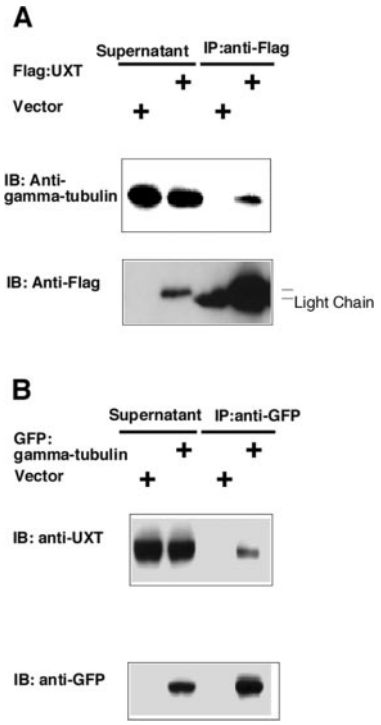


Figure 6. UXT is associated with γ-tubulin. (A) HEK293T cells were transfected with FLAG:UXT or the empty vector. Lysates were immunoprecipitated with an anti-FLAG antibody, followed by Western blot analysis using the anti-γ-tubulin antibody. Endogenous γ-tubulin is coimmunoprecipitated with FLAG:UXT. Note that the FLAG:UXT protein migrates very closely to the position of the IgG light chain on the blot. (B) HEK293T cells were transfected with γ-tubulin:GFP or GFP empty vector. Lysates were immunoprecipitated with an anti-GFP antibody, followed by Western blot analysis using an anti-UXT antibody. Note that the endogenous UXT is coimmunoprecipitated with γ-tubulin:GFP.

tubule network. Microtubule network irradiates from the cell center (centrosome) in untransfected cells but not UXT-overexpressed cells (Figure S3). To determine whether the alteration of γ-tubulin localization reflects any structural changes of the centrosome, we used electron microscopy to examine the centrosome under higher resolution. In U2OS cells transfected with EGFP (control), the centrosomes were typically intact (Figure 7B, top). In contrast, the centrosomes in EGFP:UXT-transfected cells were almost completely disassembled (Figure 7B, bottom).

Knockdown of UXT with siRNA Causes p53-independent Cell Death

To further explore the function of UXT, we performed siRNA knockdown of UXT in U2OS cells using UXT siRNA duplex. We used the following nonoverlapping target sequences for UXT siRNA: ^{362}GCA ACA GCC UCA CCA AGG A^{580} and ^{434}UAC AAG GCC UGC AGA AUU U^{452}. Similar results using both of these two oligonucleotides were obtained. The data presented here were obtained using the first oligonucleotide. The data obtained using the latter oligonucleotide are shown as supplemental data (Figure S4). To assess the potency of the siRNA for UXT, we transfected the siRNA into the U2OS cells stably expressing EGFP:UXT. It was clear that the GFP signal was knocked down in a dose-dependent manner when the siRNA concentration for UXT increased (our unpublished data). Seventy-two hours after transfection, the UXT siRNA oligo caused significant, but incomplete, reduction of UXT protein expression (Figure 8A).

We noted that there were more floating cells after UXT siRNA treatment and fewer attached cells than after control siRNA treatment (Figure 8B). Effects of siRNA on the induction of cell death were therefore quantified. When UXT protein level was knocked down by siRNA, we found that 40.2 ± 0.2% (mean ± SD) of the cells transfected with UXT siRNA and 5.6 ± 0.1% of the control cells were found in the sub-G1 population (Figure 8C). The cell death-promoting effect of UXT siRNA correlates with a decrease in both G_1 and G_2 population, indicating that UXT may be essential for the survival of human cells.

To test whether p53 plays any role in the cell death induced by UXT siRNA treatment, we transfected UXT siRNA into the p53 deficient HCT116 (p53−/−) and the parental HCT116 (p53+/+) cell line. Massive cell death was observed in both cell lines (Figure 8D). Thus, it seems that the cell death caused by UXT knockdown is not dependent on the p53 status.

DISCUSSION

To our knowledge, this is the first report to demonstrate that the prefoldinlike protein UXT represents a novel centrosomal component. We initially identified UXT as a Cdc14A binding partner. Although the functional relationship between Cdc14A and UXT is not completely understood, we have made several definitive observations concerning UXT. Our results indicate that UXT can bind to and colocalize with the well-characterized centrosomal marker γ-tubulin. Consistent with a role of UXT in the centrosome, ectopic expression of the protein can alter γ-tubulin localization. Furthermore, we provided evidence that UXT is essential for cell viability.

Immunofluorescent staining of the endogenous UXT protein revealed strong signals at the centrosome. Interestingly, the two anti-UXT antibodies generate different centrosomal staining patterns. One antibody detects UXT in the centrosome in the interphase, whereas the other only recognizes UXT disposed in the mitotic centrosome. A plausible explanation for this observation is the dynamic UXT protein disposition during the cell cycle in which UXT epitopes become obscured by interactions with other centrosomal molecules. Alternatively, UXT may be subject to posttranslational modification in a cell cycle-dependent manner, which alters the structure of UXT and thus the epitopes for the antibodies. Although further experiments are needed to distinguish these possibilities, we think that the observed change of UXT staining pattern may be used as a hallmark for the centrosome duplication events.

Because of its extensive sequence similarity to prefoldin, it is possible that UXT is a component of a protein chaperone complex. Indeed, UXT can bind to the prefoldin subunit 2 (Gstaiger *et al.*, 2003). In eukaryotes and archaea, the characterized prefoldin stoichiometry is a hexameric molecular chaperone complex built from two related classes of subunits (Siegert *et al.*, 2000). In comparison, the prefoldinlike chaperone of *Escherichia coli* consists of a homotrimer of the Skp protein (Salisbury, 2003). We have recently purified UXT using size exclusion chromatography. The UXT protein, whose predicted molecular weight is 20 kDa, migrated as a single peak with a calculated molecular weight of 120 kDa (our unpublished data). We suggest that UXT forms a homohexamer similar to other prefoldin complexes.

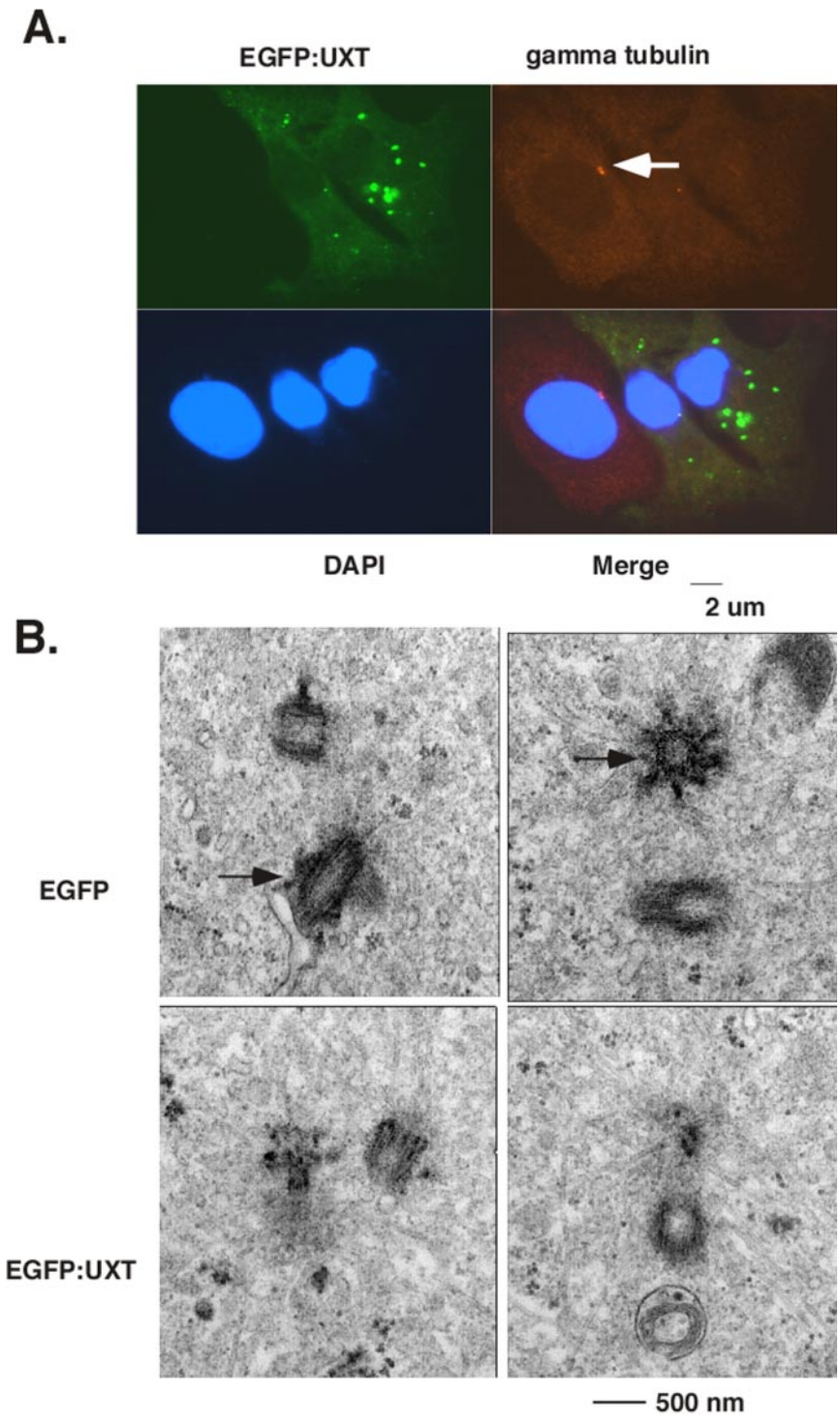


Figure 7. Overexpression of UXT disrupts the centrosome structure. (A) Loss of centrosomal γ-tubulin staining in U2OS cells after overexpression of EGFP:UXT. U2OS cells with transient expression of EGFP:UXT (green) were immunostained with the anti-γ-tubulin antibody (red). DNA was stained with DAPI. Note that the γ-tubulin staining on the centrosome is diminished in cells with over expression of GFP-UXT. (B) Electron microscopic image of disorganized centrosome in U2OS cells with overexpression of EGFP:UXT. U2OS cells, transfected with either EGFP or GFP-UXT, were processed for electron microscope imaging. Top, image of the centrosome in U2OS cells transfected with EGFP (control). Bottom, image of the abnormal centrosome in U2OS cells transfected with EGFP:UXT. The arrow indicates a normal centriole.

We have demonstrated that UXT is essential for cell viability in human cells by siRNA knockdown studies. It is noteworthy that the UXT homologue in *Caenorhabditis elegans*, the H20J04.5 protein, has also been identified as an essential gene product during the recent genome-wide gene function analysis using siRNA (Lange *et al*., 2000; Maeda *et al*., 2001). The prefoldin complex is essential for the proper folding of cytoskeletal proteins (Lundin *et al*., 2004). Inactivation of prefoldin subunits in *C. elegans* by siRNA abrogates pronuclear migration during embryogenesis, which indicates that prefoldin is required for microtubule function (Le Bot *et al*., 2003). Thus, the current data support the notion that UXT plays a crucial role in normal centrosomal biogenesis and cell survival, probably by ensuring proper folding of proteins and preventing protein aggregation in the crowded environment of the centrosome.

Our data indicate that over-expression of UXT in U2OS cells causes dislocation of the pericentriolar protein γ-tubulin. Electron microscopic studies revealed disorganized centrosomes in UXT-overexpressing cells. Thus, it seems that

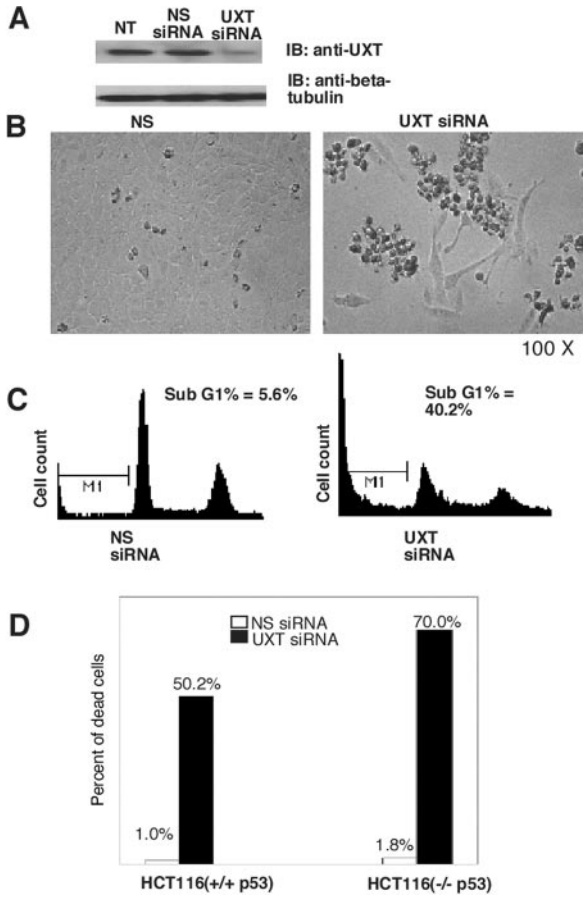


Figure 8. UXT siRNA knockdown causes cell death. Two nonoverlapping UXT siRNAs were tested and produced similar results. The data obtained with one UXT siRNA were shown. The data obtained with another UXT siRNA were shown as supplemental data (Figure S2). (A) Efficacy of siRNA knockdown of the endogenous UXT protein. U2OS cells were treated with transfection reagent (NT), nonspecific siRNA (NS siRNA), or siRNA for UXT (UXT siRNA), respectively. The cell lysates were subject to Western blot using either the anti-UXT antibody 1B2 (top) or the anti-β-tubulin antibody (bottom). The protein levels of UXT were specifically reduced after 72-h treatment with siRNA for UXT. (B) UXT knockdown inhibits cell proliferation. U2OS cells were treated with nonspecific siRNA (left) or siRNA for UXT (right). Pictures were taken 72 h later. (C) UXT knockdown leads to cell death. U2OS cells were treated with nonspecific RNA or the UXT siRNA oligos. Seventy-two hours later, all the cells were collected and used for propidium iodide staining of DNA content by fluorescence-activated cell sorting. (D) p53 is not required for cell death caused by UXT knockdown. The HCT116 (p53+/+) or p53-negative HCT116 (p53−/−) cells were treated with either the control siRNA or the UXT siRNA. Seventy-two hours after transfection, the percentage of cell death was assessed by the trypan blue assay. The diagram shows the representative results of three experiments.

overexpression of UXT causes not only dispersion of γ-tubulin but also loss of structural integrity in the centrosome. Because UXT can directly bind to the essential centrosomal protein γ-tubulin, overexpression of UXT may interfere with the γ-tubulin function, thereby impairing the organization of the centrosome. Another possibility is that, because UXT can bind to the prefoldin subunit 2 (Gstaiger *et al*., 2003), overexpressed UXT may cause disorganization of the centrosome by sequestering this subunit and disrupting the prefoldin complex that is required for the assembly of core centrosomal components, including γ-tubulin.

The current study provides additional evidence to support the hypothesis that UXT overexpression may correlate with or contribute to tumorigenesis. UXT was originally identified as a novel gene mapped to the chromosomal region Xp11, a locus linked to a variety of disease (Schroer *et al*., 1999). Because UXT is prevalent and abundant in various human tumor tissues, it was suspected to be involved in tumor development (Schroer *et al*., 1999). In our study, we have demonstrated that UXT expression is elevated in some forms of human cancers, including bladder, breast, ovarian, and thyroid cancers. In contrast to our study, it has been reported that UXT is a coactivator for the androgen receptor (Markus *et al*., 2002) and that the expression of UXT in prostate cancer is less than that in normal tissue (Taneja *et al*., 2004). In our initial screening experiment using the DiscoverLight tissue lysate sets, UXT expression level is similar between the normal prostate tissue and prostate tumor. Thus, we have not checked the expression levels UXT in prostate cancers by Western blotting. It is possible that the effects of UXT abnormality on transformation are tissue specific. It is also possible that either up-regulation or down-regulation of UXT can contribute to oncogenesis. It should also be noted that the "discrepancy" may arise from the difference between the antibody and the assays used in our study and those used in the previous study. In the study of Taneja *et al*. (2004), a polyclonal antibody preparation was used for immunostaining of cancerous or normal tissues, which may account for their results. In our study, we used a monoclonal anti-UXT antibody to detect UXT protein levels by Western blot. We think that this is a more stringent assay because it is based on both the binding of the antibody and the size of the molecule detected. Although additional experiments are necessary to substantiate the linkage between UXT levels and human cancer, we predict that UXT may be used as a marker for malignant transformation in at least some human cancers. Moreover, based on our findings that UXT is localized to the centrosome and that over-expression of UXT led to dislocation of centrosomal γ-tubulin and centrosome organization, we propose that UXT abnormality may cause dysfunction of the centrosome, thereby resulting in defects in chromosome separation that ultimately lead to aneuploidy and malignant transformation.

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