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Region Frequently Amplified in Metastasizing Breast
Cancer

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13. ABSTRACT (Maximum 200 Words) The TACC1 gene was isolated from a region of chromosome 8p11 which shows amplification in breast cancers, especially those which show metastasis to the lymph nodes. Preliminary studies showed that overexpression of TACC1 in mouse fibroblasts cause morphological transformation. We have now shown, using immunohistochemistry and immunoprecipitation, that TACC1 is present in the cytoplasm and the nucleus and furthermore that it can associate with the centrosome and mitotic spindle apparatus during mitosis. Using the full length TACC1 protein as bait to screen a human mammary epithelial cDNA library, we have identified several proteins that interact with TACC1. TACC1 interacts with the cytoskeletal and microtubule associated proteins ch-TOG and FHL3. TACC1 also associates with proteins involved in chromatin remodeling (GAS41), transcription (FHL2 and 3), and RNA splicing and degradation (L-Sm7). This suggests that TACC1 can form multiple complexes, dysregulation of which may be an important step during progression of breast tumors. Further characterization of the intracellular pathways that use TACC1 for their function will help further understand its role in breast tumorigenesis.				
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INTRODUCTION

In breast cancer, studies have identified several regions of the genome associated with breast tumor development and progression¹⁻⁴. For instance, amplifications involving 8q24, 11q13 and 17q12 are frequently observed in breast tumors⁴. In each case, the crucial genes located within these particular amplicons have been identified and encode known proto-oncogenes, MYC, CCND and ERBB2, respectively. Additional amplicons have likewise been identified, although for many of these regions, the crucial "driver" gene has yet to be identified. Amplification of the proximal short arm of chromosome 8 is observed in approximately 10-15% of breast cancer, and is particularly associated with metastasis to the axillary lymph nodes^{5,6}. However, although originally identified as a single amplicon, the rearrangement of 8p11 involved in breast cancer is significantly more complex. It is now thought that there are two separate amplicons located in this region. The first is centered upon the FGFR1 gene, and the second, more centromeric, region is associated with the PLAT gene³. In our initial analysis of the 8p11 region, we identified the novel Transforming acidic coiled coil 1 gene, TACC1⁷. TACC1 is the founding member of the evolutionarily conserved TACC family of genes⁸. These genes encode proteins that are highly acidic and contain the conserved 200 amino acid coiled coil domain, the TACC domain, which has been shown to bind a number of key regulatory molecules^{8,9}

This is the final report for this grant. In December 2000 the PI relocated his laboratory from the Cleveland Clinic to Roswell Park Cancer Institute.

BODY

Task 1: Assess the amplification and/or expression of TACC1 in breast tumors

Rearrangements of the short arm of chromosome 8 are noted in several different cancers¹⁰⁻¹². Although small critical regions of overlap have been mapped for prostate, ovarian and breast cancer, typically, the deleted or amplified regions span a significant portion of 8p21-8p11, encompassing the TACC1 locus. To determine whether changes in TACC1 gene expression could be linked to the tumorigenic processes in a number of different cancers, we first hybridized the unique 5' terminal section of TACC1 to a multiple cancer profiling array (BD Biosciences Clontech) which contains approximately 60 tumor and corresponding normal tissue control samples. As demonstrated in Fig. 1, expression of TACC1 mRNA is decreased approximately 30% of breast tumors. This suggests that **downregulation, as opposed to the predicted upregulation of TACC1 is associated with the development of breast tumors**



Fig. 1. Analysis of the expression of TACC1 mRNA using the Clontech Cancer Profiling Array. This blot contains SMART-amplified cDNA from tumor (T), and matched normal (N) tissues. The array has already been hybridized by the manufacturer to assure even sample loading. TACC1 mRNA is reduced in the tumor samples compared to their cognate normal partner. We next surveyed a series of breast cancer cell lines to determine whether they express the TACC1 protein. Western blot analysis using a previously characterized TACC1 antibody (8)

indicated that the TACC1 protein is expressed as an approximately 120kDa species in the immortal but not transformed MCF10A breast cell line (Fig. 2). When compared to MCF10A, the TACC1 protein is expressed in the majority of the cell lines, however expression levels are extremely variable, ranging from relatively low levels (MCF7 and HCC1395) to higher levels expressed in T47D, MDA-MB-468 and MDA-MB435, suggesting that the exact level of TACC1 expression could be regulated differentially in different tumor lines. These analyses alone did not answer the question as to whether the expression level of TACC1 is related to stage, malignancy, differentiation or growth potential.

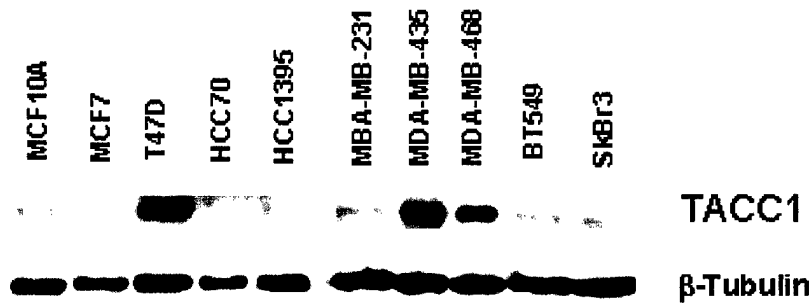


Fig. 2: Western Blot analysis of TACC1 protein expression in breast cancer cell lines. TACC1 is expressed at variable levels in different cancer cell lines. Even loading was confirmed using an antibody to β -tubulin.

Although not specifically part of this proposal, we have now performed a preliminary analysis of TACC1 protein expression in paraffin wax embedded breast tumors to determine whether the aberrations in TACC1 gene expression in cell lines was an effect of culture conditions, or was a true representation of variability of TACC1 expression in breast tumors. In a limited test set of breast tumors, we have shown that the TACC1 protein is significantly reduced in 30% of resected breast tumors (Fig 3). However, similar to the breast cancer cell lines, we also identified tumors that have a significantly higher level of expression than the normal counterpart. Although we have yet to correlate expression of TACC1 with other well characterized tumor biomarkers, recent work by other research groups has now suggested that TACC1 expression is reduced in 50% of breast tumors, and may be inversely correlated with Her2/neu expression¹³.

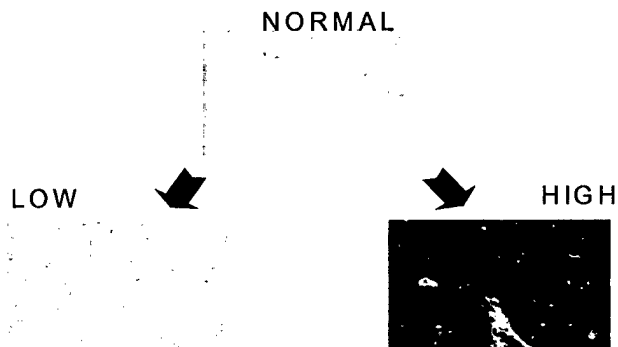


Fig. 3: TACC1 expression in three breast tumors that express normal, reduced and high levels of TACC1 protein (brown). High signal may represent alternatively spliced dominant negative transcripts, as noted in gastric cancer.

A recent report has shed light on this apparent paradox: TACC1 overexpression in gastric cancer is due to the increased expression of cancer specific transcript variants driven from two alternative promoters 5' to the originally identified promoter 1. Thus, gastric carcinomas express variants D and F, which are not expressed in the normal gastric mucosa. In addition, these transcripts are expressed at extremely

low levels, in relatively few tissues, except the brain. Significantly, this phenomenon has also been noted for the p63 gene, where amplification of the p63 locus results in overexpression of a dominant negative variant, p68^{ΔIS}, lacking the N-terminal transactivation domain in lung tumors¹⁴. Similarly, as is the case for the TACC1 splice variants D and F in gastric carcinoma, this particular p63 splice variant results in an antigenic response in cancer patients¹⁵, paving the way to potential screening strategies to detect this variant in the sera from patients. This raises the possibility that **loss of expression or alternative splicing of TACC1 in breast cancer could be an underlying factor in the development of breast cancers.**

Task 2: Determine the effect of overexpression of TACC1 in vitro:

To reconcile the apparent paradox that either loss of TACC1 or gain of expression of a TACC1 variant could result in a proliferative advantage, we hypothesized that the originally identified TACC1 transcript, TACC1A, may function as a tumor suppressor. Thus, we introduced a plasmid construct (EGTACC1A), which expresses the TACC1A open reading frame fused to the green fluorescent protein (EGFP)¹⁶ into three breast cancer cell lines. The results for MCF7 and MDA-MB-468 are shown in Fig. 4. The construct was transfected into each cell line, and stable cell lines selected as previously described (8). Expression of the fusion protein was verified by fluorescence and western blot analysis demonstrated equivalent expression of the EGFP fusion protein (data not shown). TACC1A overexpression did not adversely affect the ability of transfected MDA-MB-468 and MCF7 to proliferate (data not shown).

One of the most important indicators for a potential oncogene or tumor suppressor is the ability of the gene to impart, or abolish anchorage independent growth in vitro. We were, thus, particularly interested in determining whether transfection of TACC1A into MCF7 and MDA-MB-468 cells would alter cellular motility and growth in soft agar. Interestingly, TACC1A overexpression produced no significant alteration in the ability of MCF7 to form colonies in soft agar ($P=0.11$). However, in the case of MDA-MB-468, the number of TACC1A overexpressing colonies was significantly reduced ($P=0.01$) when compared to controls (Fig. 4). MCF7 has previously been shown to migrate poorly through a basement membrane matrix (Matrigel), and transfection of EGTACC1A into MCF7 failed to increase the efficiency of migration. However, EGTACC1A/MDA-MB-468 transfectants were significantly impaired in their ability to invade and migrate through the Matrigel matrix ($P=0.001$), suggesting that **TACC1A may act to inhibit the invasion of some breast tumor cells through the basement membrane**, and thus may link loss of TACC1A expression to the metastatic events associated with rearrangements of the short arm of chromosome 8.

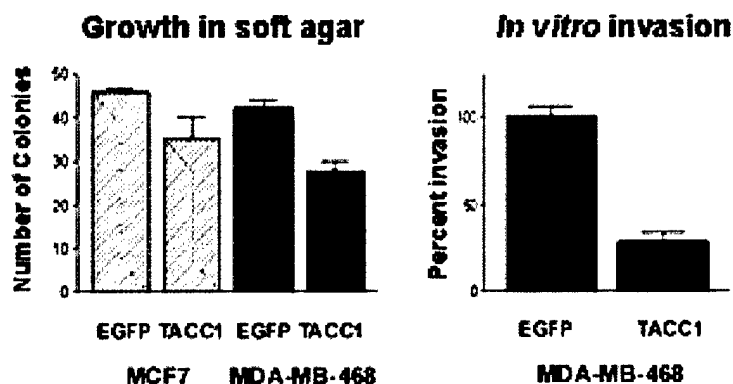


Fig. 4: In vitro growth characteristics of MDA-MB-468 and MCF7 transfected with TACC1A. The ability of MDA-MB-468 to colony formation and invasion was reduced by overexpression of TACC1A ($P<0.05$ in each case).

Task 3: Determine the subcellular localisation of the TACC1 protein

Characterizing the function of TACC1 ultimately depends on establishing its intracellular location. According to PSORT predictions, the predicted 88kD TACC1 protein contains two nuclear localization signals, NLS1 and NLS2. This suggested, but did not prove that TACC1 is localized to the nucleus. In an article published in the Proceedings of the National Academy of Sciences, we determined the normal subcellular distribution of the human TACC proteins using EGFP-tagged proteins and by standard immunohistochemical stains using antibodies generated to the TACC proteins¹⁶. During interphase, the TACC proteins are distributed throughout the cell, with the TACC1A splice variant preferentially located in the nucleus. However in mitotic HeLa and primary fibroblasts, the TACC1 stains the mitotic spindle and the centrosomes to varying extents. Transient overexpression of the TACC proteins results in the formation of large polymers in the cytoplasm, which retain the ability to bind to microtubules in a regulated manner. This accumulation does not occur in the absence of the TACC domain, suggesting that the TACC proteins interact with microtubules either directly or indirectly through the conserved TACC coiled coil domain. However it is unclear whether the main role of the TACC proteins is in the organization of the microtubule network, or that TACC proteins perform additional functions distinct from their association with microtubules.

Task 4 Yeast two hybrid screening of breast tumor cDNA libraries

A significant improvement of our understanding of the function of TACC1 in the normal cells has come from identifying potential protein factors that interact with TACC1. Our initial search of potential TACC1 interacting proteins used an adult cDNA library, derived from bone marrow. This screen identified two known genes, SIAH1 and GAS41, two proteins implicated in potential growth control pathways in different cell types. Both of these proteins are expressed in the normal breast tissue.

Recently a commercial mammary gland cDNA library (Clontech) has become available which has allowed us to identify TACC1 binding proteins in normal breast tissue. Our initial screen of one million cDNA clones from this library identified sixty-nine clones by nutrient selection. Encouragingly, a proportion of clones represented the SIAH1 and GAS41 proteins described above, suggesting that these proteins do interact with TACC1 in both normal breast tissue, and potentially in breast tumors (see below). In addition to these proteins, we also identified four other proteins of known function, including ch-TOG, a protein essential for mitotic spindle assembly, which is overexpressed in hepatic and colon cancers (published in the *Biochemical Journal*), and the human orthologue of p16, a bovine protein involved in transport of proteins from the endoplasmic reticulum to the golgi apparatus prior to sorting to different subcellular compartments. The second protein identified, L-Sm7 is a component of a protein complex assembled in the cytoplasm and transported to the nucleus. This complex is a key component in splicing of pre-mRNAs. We have mapped the precise location of the binding site in TACC1 for these proteins by using smaller sections of the TACC1 cDNA and carrying out yeast two-hybrid analysis. The binding site for p16, and LSm-7 partially overlap those of GAS41 and SIAH1, suggesting that these proteins could compete with each other to bind TACC1. The fourth protein identified was FHL3, a member of the LIM domain family of proteins. FHL3

interacts with CtBP2¹⁷, and cdc25B¹⁸, further implicating TACC1 in transcriptional and cell cycle control.

Task 5: Verify TACC interacting protein clones by coimmunoprecipitation

The interactions between TACC1, GAS41 and the FHL proteins have been verified by coimmunoprecipitation¹⁹. GAS41 has recently been shown to bind to the nuclear matrix protein NuMA, a protein that is critical to the formation of the mitotic spindle. We have also shown that TACC1 binds weakly to the mitotic spindle during cellular division¹⁶, but have been unable to demonstrate a direct interaction between NuMA and TACC1 by coimmunoprecipitation. As NuMA only interacts weakly with GAS41 in mammalian cells²⁰, TACC1 may be the preferential GAS41 binding partner in interphase cells. In addition, GAS41 has recently been shown to be a component of the SWI/SNF chromatin remodelling machinery²¹. Together with the observation of a direct interaction between FHL3, and its related family member FHL2 in human cells, this could implicate TACC1 in scaffolding events important at multiple stages of gene regulation.

Task 6: Screen cDNA libraries to isolate full length cDNA clones

Our original task 6 was to screen cDNA libraries to identify full length genes interacting with TACC1. This task has largely been unnecessary since all of the genes showing interactions are available as full length sequences. Smaller constructs have been isolated to facilitate refined mapping of the binding domains on these proteins.

KEY RESEARCH ACCOMPLISHMENTS:

Generation of a polyclonal antibody against TACC1

Observation of altered regulation, and potential splice variants of TACC1 associated with breast cancer.

Characterization of the tumor suppressor activity of the TACC1A splice variant

Demonstration that TACC1 interactions with the SWI/SNF component GAS41.

Identification FHL3 as a TACC1 binding protein, implicating TACC1 in cell cycle and transcriptional control.

Implication of TACC1 as an RNA maturation and transport protein through an interaction with LSm7.

REPORTABLE OUTCOMES:

Development of cell lines overexpressing TACC1A

Generation of a polyclonal antibody against TACC1

Publications:

Gergely F, Karlsson C, Still I, Cowell JK, Kilmartin J, Raff J. (2000) The TACC domain identifies a family of centrosomal proteins that can interact with microtubules. *Proc Natl Acad Sci* 97; 14352-14357.

Lauffart,B.; Howell,S.J.; Tasch,J.E.; Cowell,J.K.; Still,I.H. (2002) Interaction of the transforming acidic coiled-coil 1 (TACC1) protein with ch-TOG and GAS41/NuBII suggests multiple TACC1-containing protein complexes in human cells. *Biochem. J.* 363: 195-200.

CONCLUSIONS

In conclusion, dysregulation, either as a result of loss of expression, or possibly overexpression of variant isoforms is a significant event in breast cancer. A functional explanation of this may lie in the ability of TACC1 to form multiple different protein complexes in the cell. In the cytoplasm, it appears to act as scaffolding/bridging proteins important for centrosomal function. TACC1 may also perform a similar function in the nuclear matrix, by acting as a scaffold or bridging protein between transcription factors and basal transcription initiation complexes. Therefore, regulation of the dynamic interaction between TACC1 and its interacting factors may be critically important to the control of division of normal and malignant breast epithelial cells.

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Appendix

Gergley F, Karlsson C, Still I, Cowell JK, Kilmartin J, Raff J. (2000) The TACC domain identifies a family of centrosomal proteins that can interact with microtubules. *Proc Natl Acad Sci* 97; 14352-14357.

Lauffart,B.; Howell,S.J.; Tasch,J.E.; Cowell,J.K.; Still,I.H. (2002) Interaction of the transforming acidic coiled-coil 1 (TACC1) protein with ch-TOG and GAS41/NuBI1 suggests multiple TACC1-containing protein complexes in human cells. *Biochem. J.* 363: 195-200.

The TACC domain identifies a family of centrosomal proteins that can interact with microtubules

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We recently showed that the *Drosophila* transforming acidic coiled-coil (D-TACC) protein is located in the centrosome, interacts with microtubules, and is required for mitosis in the *Drosophila* embryo. There are three known human TACC proteins that share a conserved, C-terminal, coiled-coil region with D-TACC. These proteins have all been implicated in cancer, but their normal functions are unknown. We show that all three human TACC proteins are concentrated at centrosomes, but with very different characteristics: TACC1 is weakly concentrated at centrosomes during mitosis; TACC2 is strongly concentrated at centrosomes throughout the cell cycle; and TACC3 is strongly concentrated in a more diffuse region around centrosomes during mitosis. When the C-terminal TACC domain is overexpressed in HeLa cells, it forms large polymers in the cytoplasm that can interact with both microtubules and tubulin. The full-length TACC proteins form similar polymers when overexpressed, but their interaction with microtubules and tubulin is regulated during the cell cycle. At least one of the human TACC proteins appears to increase the number and/or stability of centrosomal microtubules when overexpressed during mitosis. Thus, the TACC domain identifies a family of centrosomal proteins that can interact with microtubules. This may explain the link between the TACC genes and cancer.

centrosome | cancer | mitosis

Both centrosomes and the microtubules they organize play crucial roles in many cell processes (1–3). Despite their importance, however, surprisingly little is known about how centrosomes interact with microtubules at the molecular level.

Considerable progress has been made recently in understanding how γ -tubulin ring complexes in the centrosome are involved in microtubule nucleation (4, 5). The interaction between centrosomes and microtubules, however, appears to be more complicated than just a simple nucleation (6–9). To understand better how centrosomes interact with microtubules, we and others have biochemically isolated a number of proteins from *Drosophila* embryos that interact with microtubules *in vitro* and concentrate at centrosomes *in vivo* (10–13). We have previously shown that one of these proteins, *Drosophila* transforming acidic coiled-coil (D-TACC), is essential for mitotic spindle function in the early *Drosophila* embryo (14). In embryos where D-TACC function is perturbed, spindle and astral microtubules are abnormally short and weak, and this leads to failures in nuclear migration and chromosome segregation.

The C-terminal region of D-TACC is predicted to form a coiled-coil that is similar to that found in the mammalian TACC-containing proteins. The normal functions of the three known mammalian TACC proteins are unknown, but several observations suggest that the proteins may contribute to cancer: the human TACC genes are all in genomic regions that are rearranged in certain cancer cells; TACC3 is up-regulated in some cancer cell lines; and the overexpression of TACC1 transforms mouse fibroblasts (15, 16). Very recently, TACC2 has also been identified as a potential tumor suppressor protein called AZU-1; the expression of the protein is down-regulated in many breast carcinoma cell lines

and primary tumors, and restoring TACC2/AZU-1 protein to normal levels reduces the malignant phenotype of cells both in culture and *in vivo* (17). A recently identified *Xenopus* protein called maskin, which is related to TACC3, has been shown to be involved in regulating the translation of specific mRNAs in the developing frog embryo (18).

We previously showed that the conserved C-terminal region (which we call the TACC domain) of D-TACC can direct a heterologous fusion protein to centrosomes and microtubules in *Drosophila* embryos (14). Moreover, we showed that the human TACC2 protein is also concentrated at centrosomes in human cells. We therefore postulated that all of the TACC proteins might interact with microtubules and be concentrated at centrosomes via their TACC domains. Here, we provide evidence that this is the case. We show that human TACC1 and TACC3 are concentrated at centrosomes, although only during mitosis. We demonstrate that, when the TACC domain is overexpressed in human cells in culture, it forms large polymeric structures in the cytoplasm that can interact with both microtubules and tubulin. When the full-length TACC proteins are overexpressed, similar polymers form, but their interaction with microtubules and tubulin is now cell-cycle regulated. Moreover, in cells that overexpress the TACC3 protein, the number of centrosomal microtubules appears to be increased. These findings suggest that the TACC domain is a conserved motif that can interact with centrosomes and microtubules and that the TACC proteins may play a conserved role in organizing centrosomal microtubules.

Materials and Methods

TACC cDNAs. The sequences of the TACC cDNAs have the following GenBank accession numbers: AFO49910 (TACC1); AFO95791 (TACC2); and AF093543 (TACC3). The numbering of amino acids refers to the predicted proteins encoded by these cDNAs. Recently, we identified a larger TACC2 cDNA that encodes a larger protein of 1,026 aa. In preliminary experiments, this larger protein behaved in a very similar manner to the shorter protein (not shown).

Antibody Production. Antibodies were raised in rabbits against bacterially expressed and purified glutathione *S*-transferase (19) or maltose-binding protein (New England BioLabs) fusion proteins that contained the following regions of the TACC proteins:

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: D-TACC, *Drosophila* transforming acidic coiled-coil; GFP, green fluorescent protein.

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TACC1, amino acids 1–323; TACC2, amino acids 689–1026; and TACC3, amino acids 73–265. Rabbit antisera were raised by Eurogentec (Brussels), and we affinity purified and stored the antibodies as described (20).

Immunofluorescence. Human HeLa or primary fibroblast cells (MHF 181 primary foreskin fibroblasts) were cultured, fixed with methanol, and stained with antibodies as described (21). All affinity-purified primary antibodies were used at 1–2 $\mu\text{g/ml}$. DM1A anti- α -tubulin antibody and GTU-88 anti- γ tubulin antibody (Sigma) were used at 1:500 dilution. Appropriate Cy5-, Cy3- (Jackson), or Alexa488- (Molecular Probes) coupled secondary antibodies were used at 1:500 dilution. With the transfected cells, we performed several control experiments to eliminate the possibility that the fluorescence from the green fluorescent protein (GFP)-fusion proteins was “bleeding through” into other channels. All imaging was performed by using a Bio-Rad 1024 scanning confocal microscope. Images were imported into Adobe PHOTOSHOP.

Western Blotting. Whole-cell extracts were made by pelleting HeLa cells and boiling the pellet in SDS sample buffer. The extracts were separated by SDS/PAGE and blotted to nitrocellulose as described (22, 23). Blots were incubated with primary antibodies at 1–2 $\mu\text{g/ml}$ final concentration, and antibody binding was detected by using a Supersignal kit (Pierce), according to the manufacturer’s instructions.

DNA Constructs. Transient transfection experiments were performed with various regions of the TACC proteins subcloned into either pEYFP or pEGFP vectors (CLONTECH), producing YFP-TACC or GFP-TACC fusion proteins. Several regions of the TACC proteins were also subcloned into the pCMV-Tag2 (Stratagene), pSEM, or pCDNA3 (Invitrogen) vectors, producing either FLAG-TACC fusion proteins or untagged TACC proteins. Similar results were obtained with the different regions of the TACC proteins whether they were expressed on their own or as fusion proteins with GFP or FLAG, except that the GFP-fusion proteins routinely appeared to be expressed at higher levels. The following regions of the TACC proteins were used (numbers for TACC2 refer to the recently discovered larger TACC2 cDNA): full-length TACC proteins (TACC1, amino acids 1–805; TACC2, 397–1026; TACC3, 1–838); TACC domains (TACC1, amino acids 588–804; TACC2, 771–1026; TACC3, 604–838); TACC proteins missing the TACC domain (TACC1, amino acids 3–562; TACC2, 452–765; TACC3, 1–593).

Transient Transfection and Drug Treatment. The mammalian Profection transfection kit (Promega) was used according to the manufacturer’s instructions. In the case of drug treatments, taxol was added to 2 μM and nocodazole to 1 μM for 4–26 h, either immediately after the cells had been transfected or for 12 h before the cells were transfected (in which case the whole transfection procedure was carried out in the presence of the drug). Similar results were obtained using either protocol. To

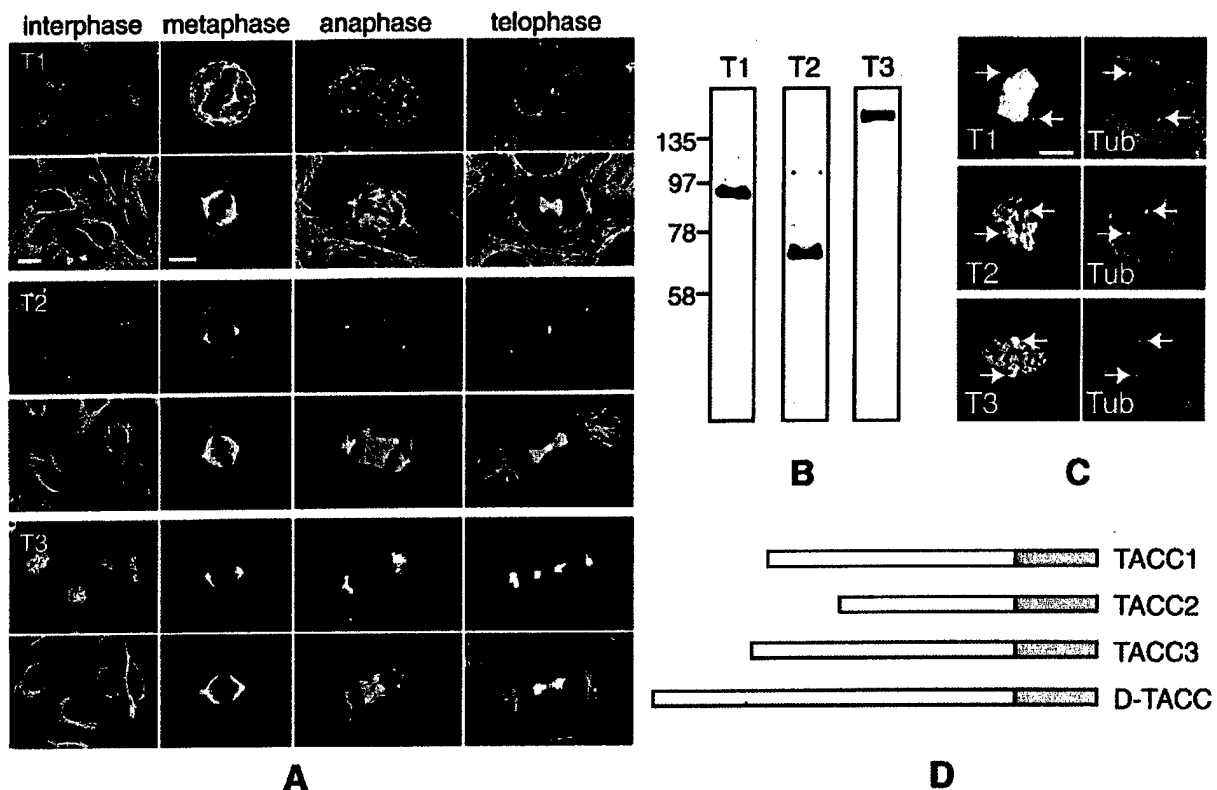


Fig. 1. A comparison of the known human TACC proteins. (A) Fixed HeLa cells at interphase, metaphase, anaphase, and telophase were stained with anti-TACC antibodies (Top; red in color panels) and anti-tubulin antibodies (green in color panels). In this panel, and in all subsequent panels, T1 denotes TACC1, T2 denotes TACC2, and T3 denotes TACC3. Scale bars: interphase, 10 μm ; mitotic cells, 5 μm . (B) Western blots of HeLa cell extracts probed with affinity-purified anti-TACC1 (lane 1), anti-TACC2 (lane 2), and anti-TACC3 (lane 3) antibodies. (C) Nocodazole-treated cells were stained with the anti-TACC antibodies (Left) and with a mixture of anti- α -tubulin and anti- γ -tubulin antibodies (Right) to monitor the location of the centrosomes and to confirm that microtubules were depolymerized. All of the TACC antibodies stained the centrosomes (arrows) even though there were no visible microtubules in the cell. Note that the chromosomes in these cells were counterstained with propidium iodide, and the signal from this fluorophore “bleeds through” into the channel used to detect the TACC antibodies. (Scale bar = 4 μm .) (D) A schematic diagram of the TACC proteins. The shaded boxes represent the conserved 200-aa TACC domain that is predicted to form a coiled-coil.

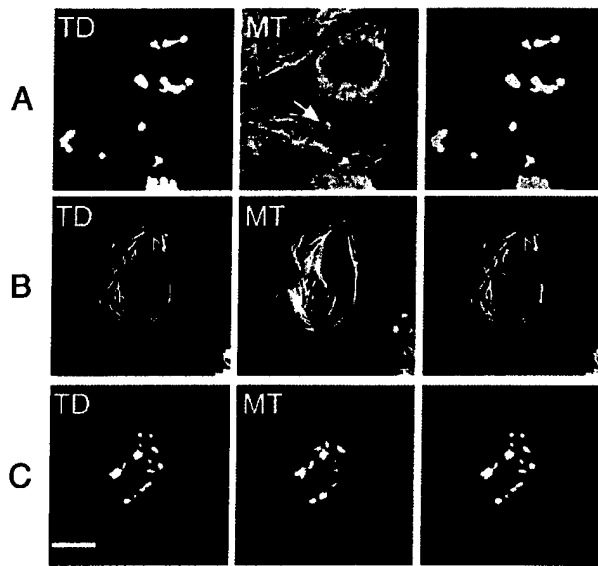


Fig. 2. The behavior of the overexpressed TACC domains is shown in normal cells (*Top*) and in cells where microtubules are stabilized by taxol (*Middle*) or depolymerized by nocodazole (*Bottom*). The TACC domain is visualized by the fluorescence of the GFP tag (*Left*). Microtubules were stained with anti-tubulin antibodies (*Center*). A merged image is shown (*Right*): in this and all subsequent merged panels, the GFP-TACC fusion protein is shown in green and tubulin in red. The arrow highlights the weak association of tubulin with a TACC domain structure in an untreated cell. In nocodazole-treated cells, the unpolymerized tubulin is concentrated around the TACC domain structures, whereas in taxol the structures stretch out along the microtubule bundles. Only the TACC2 TACC domain is shown, as the TACC domains of TACC1 and TACC3 behave identically. (Scale bar = 10 μm .)

observe cells in mitosis, we synchronized HeLa cells in G2 with a double thymidine block. The cells were transfected during the last 6–12 h of the second block and fixed 12–14 h later, when the majority of the cells should have been in mitosis. To test whether microtubules were required to maintain the localization of the TACC proteins at the centrosomes, cells were synchronized in G2 and then released for 12 h; nocodazole was then added to 25 μM for 1 h before fixation.

Electron Microscopy. Cells were fixed and processed for thin-section electron microscopy and thin-section immunoelectron microscopy as described (24).

Results

The TACC Proteins Are Differentially Distributed in the Cell. The three human TACC proteins are related to each other in a ≈ 200 -aa region. This TACC domain region is predicted to form a coiled-coil (Fig. 1D; see figure 7 in ref. 14 for a sequence alignment and coiled-coil predictions of the TACC domains). Although the three known human TACC genes appear to have been generated by gene duplication events, there is very little amino acid homology between the TACC proteins outside of the TACC domain (15, 16). We previously showed that TACC2 is strongly associated with centrosomes, and more weakly with mitotic spindles, in both HeLa cells and primary fibroblasts (14). To investigate the localization of human TACC1 and TACC3, we raised and affinity-purified antibodies against both of these proteins. In Western blots of HeLa cell extracts, the anti-TACC1 antibodies recognized a major band of ≈ 85 kDa, the anti-TACC2 antibodies a major band of ≈ 73 kDa, and the anti-TACC3 antibodies a major band of ≈ 150 kDa (Fig. 1B).

In contrast to TACC2 antibodies, the TACC1 and TACC3 antibodies did not stain centrosomes in interphase HeLa cells.

Instead, they diffusely stained both the cytoplasm and the nucleus, with TACC3 being slightly concentrated in the nucleus (Fig. 1A). In mitotic HeLa cells, however, all of the TACC antibodies stained centrosomes to varying extents: the TACC1 antibodies weakly stained centrosomes, the TACC2 antibodies strongly stained centrosomes, whereas the TACC3 antibodies strongly stained a more diffuse region around the centrosomes. All of the antibodies stained the mitotic spindle to varying extents (TACC3 antibodies being the strongest and TACC1 antibodies the weakest), and they also stained a ring-like structure at the cleavage furrow during cytokinesis. In primary fibroblasts, all three TACC proteins had a similar distribution to that shown here in HeLa cells (not shown).

To test whether the concentration of the TACC proteins at the centrosome during mitosis required microtubules, we treated HeLa cells with nocodazole to depolymerize the microtubules. All three of the TACC proteins remained concentrated at centrosomes in the treated mitotic cells (Fig. 1C), suggesting that microtubules are not required to maintain their localization at the centrosome. Interestingly, even in the absence of microtubules, the TACC proteins appeared to maintain their characteristic association with centrosomes: TACC1 was only weakly concentrated at centrosomes, TACC2 was strongly concentrated at centrosomes, and TACC3 was strongly concentrated in a more diffuse region around the centrosome.

The TACC Domain Forms Large Polymers in the Cytoplasm. We showed previously that the TACC domain of D-TACC can direct a heterologous fusion protein to centrosomes and microtubules in *Drosophila* embryos (14). We therefore overexpressed the TACC domain of each human TACC protein as a GFP fusion to assess its contribution to localizing the TACC proteins to centrosomes and spindles. Surprisingly, all of the expressed TACC domains assembled into large structures in the cytoplasm in virtually all of the transfected HeLa cells in which they were expressed (Fig. 2A). Similar structures formed when the *Drosophila* TACC domain was expressed in HeLa cells or in a *Drosophila* cell line (not shown). These structures did not form in HeLa cells when we expressed GFP on its own or in GFP fusion proteins containing the full-length TACC proteins that lacked the C-terminal TACC domain (not shown).

When we examined these structures by thin-section electron microscopy, we found that they were not random aggregates of protein but were highly ordered polymeric structures consisting of many layers of a regularly spaced electron-dense matrix (Fig. 3). Interestingly, the interlayer spacing of these polymers is about 35 nm, which is close to the expected length of the TACC domains coiled-coil (approximately 30 nm for a 200-aa α -helix).

The TACC Domain Can Interact with Microtubules and Tubulin. As the TACC polymers are easily recognizable, they provide an excellent system to study interactions between the TACC proteins and other cellular components in living cells. When we overexpressed the TACC domains in HeLa cells, the microtubule cytoskeleton was usually not dramatically perturbed, and the TACC domain polymers did not appear to interact significantly with microtubules (Fig. 2A). When we treated the transfected cells with taxol to produce large bundles of stabilized microtubules, however, the usually rounded TACC polymers often reorganized into large rod-like fibers that stretched out along the microtubule bundles (Fig. 2B). Thus, the TACC domain polymers can interact with microtubules under certain conditions.

In addition, we noticed that in nontaxol-treated cells that overexpressed a TACC domain, small amounts of tubulin appeared to accumulate around the periphery of the polymer structures (Fig. 2A, arrow). To investigate further this potential interaction with unpolymerized tubulin, we treated the transfected cells with nocodazole to depolymerize the microtubules.

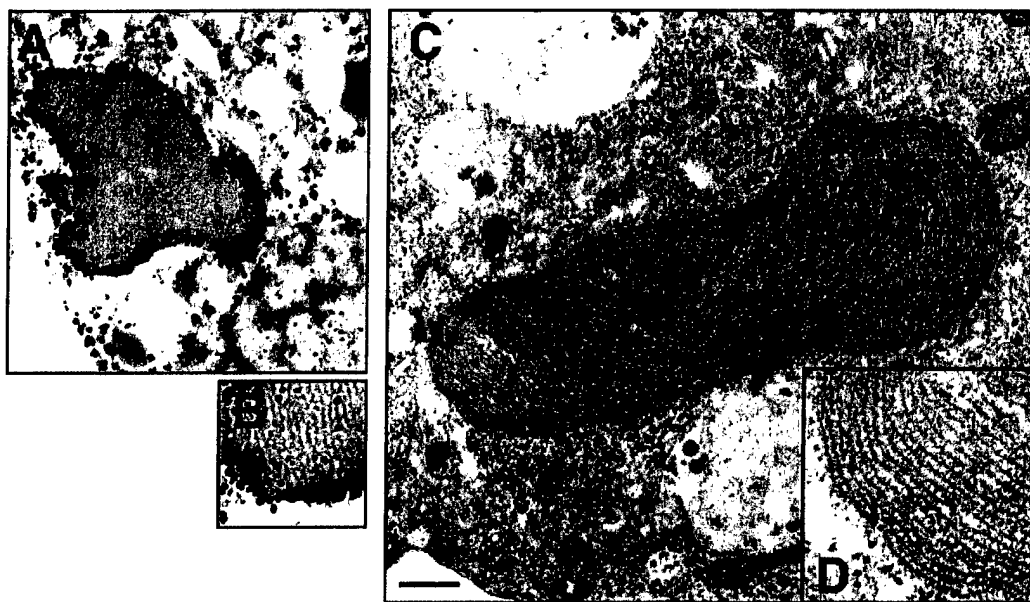


Fig. 3. The large TACC-containing structures are highly ordered polymers. (A) Immuno-electron microscopy of cells overexpressing the GFP-TACC domain fusion proteins. Cells were fixed with formaldehyde and stained with anti-GFP antibodies, followed by Nanogold-labeled secondary antibodies and silver intensification. In thin sections, the silver particles stain the edges of large, globular, cytoplasmic structures that were composed of a regularly spaced, electron dense, polymer (the cell shown here is overexpressing the TACC2 TACC domain). The staining is largely confined to the margins of these structures probably because the formaldehyde fixation has highly cross-linked the structures, impeding internal antibody access. A 2 \times higher magnification view of part of this structure is shown in B. The ordered morphology of these structures is better preserved in glutaraldehyde-fixed cells shown in C and, at 2 \times higher magnification, in D. This cell is expressing a GFP full-length TACC2 fusion protein, and the largest diameter of this TACC structure is about half the size of this cell's nucleus. (Scale bar = 0.5 μ m.)

In these cells, both anti- α (Fig. 2C) and anti- β -tubulin (not shown) antibodies strongly stained the periphery of the TACC domain polymers, suggesting that the depolymerized tubulin dimers could interact with the TACC domain polymers. We obtained similar results when microtubules were depolymerized by cooling (not shown). No interactions were detected between the polymers and γ -tubulin in either normal or drug-treated cells (data not shown), suggesting that this interaction with tubulin/microtubules is specific.

We have attempted to investigate the interaction between the TACC domain and microtubules/tubulin *in vitro* by using purified components. In both microtubule spin-down and gel-filtration experiments, however, we were unable to detect any interaction between the purified TACC domains and microtubules or tubulin (not shown). We believe that the TACC domain proteins interact with microtubules in a complex with at least one other protein (see *Discussion*).

The Interaction of the TACC Domain with Microtubules and Tubulin Depends on Its Protein Context. We next overexpressed the full-length TACC proteins as GFP fusions in HeLa cells to test whether they, too, could form similar cytoplasmic polymers. All of the full-length TACC proteins formed large structures in the cytoplasm (Fig. 4 *Left*), and thin-section electron microscopy of TACC2-expressing cells revealed that these polymers had a very similar organization to the overexpressed TACC domain polymers (Fig. 3 C and D). Unlike the TACC domains on their own, however, the morphology of these TACC structures varied somewhat between the different TACC proteins; the TACC1 structures in particular were often less compacted than the other TACC structures, and they were almost always clustered around the nucleus. Similar polymers formed when the full-length TACC proteins were expressed without the GFP tag or as FLAG-tagged fusion proteins (not shown).

The presence of the polymers again provided an opportunity

to analyze the interactions between the TACC proteins and the microtubule cytoskeleton. Whereas the majority of cells transfected with TACC1 or TACC3 had a normal-looking microtubule cytoskeleton, those overexpressing TACC2 were often rounded and had a slightly disorganized microtubule network. Moreover, the TACC2 polymers accumulated more tubulin in or around them than did TACC1 and TACC3 polymers (Fig. 4 *Left*). This difference was even more apparent in nocodazole-treated cells where most of the cytoplasmic tubulin appeared to be sequestered around the TACC2 polymers, whereas the TACC3 polymers interacted more weakly with tubulin, and the TACC1 polymers did not appear to interact with tubulin at all (Fig. 4 *Center*). In taxol-treated cells, only the overexpressed TACC2 protein polymers spread out along the taxol-stabilized microtubules; the TACC1 and TACC3 polymers did not appear to significantly interact with microtubules in these cells (Fig. 4 *Right*).

The Interaction of the TACC3 Polymers with Microtubules Is Cell Cycle Regulated, and TACC3 Appears to Stabilize Centrosomal Microtubules in Mitosis.

As only the endogenous TACC2 protein is normally present in centrosomes in interphase cells, it may not be surprising that TACC1 and TACC3 polymers failed to interact with tubulin in interphase cells. To test whether the TACC polymers could interact with microtubules in mitotic cells, we transfected synchronized HeLa cells with full-length TACC constructs and fixed them at a time point when they should have been in mitosis (Fig. 5A). Although many of the nontransfected cells were found to be in mitosis at the time of fixation, very few of the transfected cells were in mitosis. This suggests that the presence of the TACC polymers may affect cell cycle progression. We suspect, however, that the presence of these huge TACC polymers could indirectly perturb many cellular processes, and indeed all of the transfected cells die within 4–5 days of transfection. Thus, we cannot

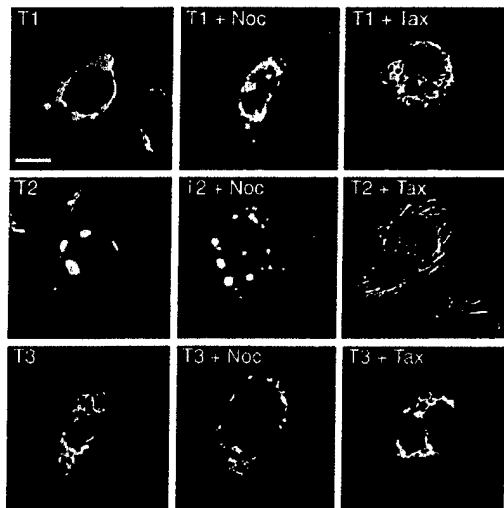


Fig. 4. The behavior of the overexpressed full-length TACC proteins. In normal transfected cells (*Left*), all of the overexpressed TACC proteins (green) form large structures in the cytoplasm. Tubulin is shown in red in the merged images; when tubulin is concentrated around the TACC structures, they appear to be yellow. Tubulin is not highly concentrated around the TACC1 or TACC3 polymers but is concentrated around the TACC2 polymers. In nocodazole-treated cells (*Center*), the TACC1 polymers do not interact with tubulin and TACC2 polymers strongly interact with tubulin, whereas the TACC3 polymers weakly interact with tubulin. In taxol-treated cells (*Right*), only the TACC2 polymers interact with the stabilized microtubules. (Scale bar = 10 μm .)

conclude that the overexpression of the TACC proteins directly affects cell cycle progression.

In the few mitotic cells that overexpressed the TACC3 protein, the polymers were less compacted than in interphase cells, and the protein was strongly concentrated in a diffuse region around the spindle poles in a manner similar to the endogenous TACC3. Strikingly, in the mitotic cells that overexpressed the TACC3 protein, there appeared to be many more microtubules associated with the spindles when compared with nontransfected cells on the same coverslip that were at similar stages of mitosis (Fig. 5B). This suggests that the extra TACC3 may increase the number and/or stability of centrosomal microtubules. In the rare mitotic cells that overexpressed TACC1, however, the polymers did not detectably associate with tubulin or centrosomes (not shown). Similarly, the TACC2 polymers remained compacted throughout mitosis and did not associate with the centrosomes, although tubulin was still strongly concentrated in or around these polymers (Fig. 5A). Although the number of transfected cells in mitosis was small, there did not appear to be any obvious delay at any particular stage of mitosis in cells transfected with any of the TACC proteins.

Discussion

The TACC Proteins Are a Conserved Family of Centrosome- and Microtubule-Interacting Proteins. We previously showed that the D-TACC protein interacted with microtubules in *Drosophila* embryo extracts and was concentrated at centrosomes in embryos. We also showed that the conserved C-terminal region of D-TACC could target a heterologous fusion protein to centrosomes and microtubules, and that the human TACC2 protein was concentrated at centrosomes in human cells. We proposed that the TACC domain was a conserved microtubule- and centrosome-interacting domain.

The data we present here support this proposal, although the three known human TACC proteins appear to interact with centrosomes and microtubules in unique ways. Unlike TACC2, both TACC1 and TACC3 are not concentrated at centrosomes

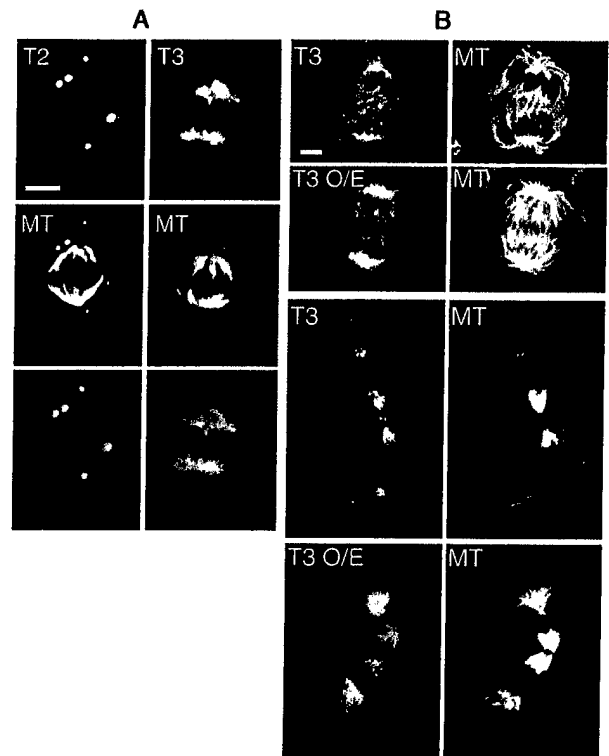


Fig. 5. The behavior of overexpressed TACC2 and TACC3 in mitotic cells. (A) The TACC2 polymers (green in merged image) remain highly compacted throughout mitosis but strongly interact with the unpolymerized tubulin (red in merged image) in the cell. During mitosis, the TACC3 polymers are much less compacted than in interphase, and they are strongly concentrated around the poles of the mitotic spindle. (Scale bar = 5 μm .) (B) TACC3 appears to increase the number of centrosomal microtubules. A comparison between transfected cells stained with anti-TACC3 (*Left*) and anti-tubulin antibodies (*Right*). In both anaphase (*Upper*) and telophase (*Lower*), the centrosomes in TACC3 overexpressing cells appear to be associated with many more microtubules. Note that all of these images were taken from transfected and nontransfected cells on the same coverslip with identical settings on the confocal microscope so that meaningful comparisons could be made between them.

in interphase but are distributed in the cytoplasm and nucleus, with TACC3 being concentrated in the nucleus of many cells. In mitosis, all three TACC proteins interact with centrosomes and microtubules but in different ways: TACC1 is only weakly concentrated at centrosomes and on spindles, TACC2 is strongly concentrated at centrosomes and more weakly associates with spindles, whereas TACC3 is strongly concentrated in a more diffuse region that surrounds the centrosome, and it has the strongest interaction with the mitotic spindle. An interaction with microtubules does not, however, appear to be required to maintain the association of the TACC proteins with centrosomes, as they remain concentrated at centrosomes even in the presence of microtubule depolymerizing agents. Thus, the TACC proteins all appear to be genuine centrosomal proteins (25) that can also associate with microtubules.

It is not clear how the TACC domain interacts with centrosomes or microtubules. We have so far been unable to observe a strong interaction between any of the bacterially expressed and purified TACC domains and purified tubulin (ref. 14 and M. J. Lee and J.W.R., unpublished observations). In the case of D-TACC, however, the bacterially expressed TACC domain binds strongly to microtubules when it is mixed with embryo extracts (14). Our preliminary data suggest that D-TACC binds to microtubules in a complex with at least one other protein and

that this is also true of the human TACC proteins (M. J. Lee, F.G., and J.W.R., unpublished observations). Surprisingly, however, if we perform similar microtubule spin-down experiments with mitotic or interphase HeLa cell extracts, we do not detect a strong interaction between any of the endogenous TACC proteins and microtubules (F.G., unpublished observations). This may represent a real difference between the human TACCs and D-TACC; as in mitotic HeLa cells treated with taxol, many supernumary asters form in the cytoplasm, but the TACC proteins do not interact with these asters and remain concentrated around the centrosomes (F.G., unpublished observations). Thus, the endogenous human TACC proteins may only associate strongly with microtubules when they are in the context of the centrosome or spindle.

The TACC Proteins Can Form Large Polymers. When the TACC proteins are overexpressed in HeLa cells, they all form large structures in the cytoplasm. Ultrastructural analysis of these structures revealed that they were highly ordered polymers, consisting of interwoven layers of a regularly spaced, electron-dense, matrix. The formation of these polymers is TACC domain dependent; fusion proteins that lack the TACC domain do not form these structures, and the TACC domain from any of the human TACC proteins (and also from D-TACC) is sufficient to drive the formation of similar polymers when overexpressed in HeLa cells. Surprisingly, the overexpressed TACC domain polymers do not normally appear to interact with centrosomes or microtubules in transfected cells. We suspect that this is because transient transfection leads to a massive overexpression of the TACC domain proteins, and this drives the formation of large, compact cytoplasmic polymers that may be unable to interact efficiently with centrosomes or microtubules. In support of this possibility, when microtubules are stabilized with taxol, the TACC domain polymers reorganize and spread out along the bundles of stabilized microtubules, perhaps because the bundles of microtubules give the compacted polymers a greater surface area of interaction.

Although the large polymers formed in transfected cells are clearly a consequence of overexpression, the ability to form such polymers is highly unusual. Several components of the yeast spindle pole body, however, form large polymeric structures when overexpressed, and their ability to polymerize is thought to be important for their function (24, 26, 27). The essential vertebrate spindle component NuMA has also been shown to form large rod-like polymers when overexpressed in human cells (28). Further experiments will be required, however, to determine whether the TACC proteins normally form polymeric structures within cells.

The Functions of the TACC Proteins. The results presented here raise the possibility that the human TACC proteins and D-TACC could perform similar functions. When, for example, TACC3 is overexpressed in cells, it is strongly concentrated around centrosomes in mitosis, and the centrosomes appear to associate with more microtubules than normal. We observe similar effects when D-TACC is overexpressed in *Drosophila* embryos (J.W.R. and K. Jeffers, unpublished work). This effect is not, however, seen in mitotic cells that overexpress TACC1 or TACC2. This may be because the large cytoplasmic polymers formed by the overexpressed TACC1 and TACC2 proteins cannot fulfil the normal functions of the endogenous proteins. Unlike the TACC3 polymers, TACC1 and TACC2 polymers remain compacted throughout mitosis, and they do not mimic the localization of the endogenous proteins. On the other hand, our results indicate that the three human TACC proteins interact with centrosomes and microtubules in different ways, suggesting that they could perform distinct and potentially nonredundant functions in cells.

If the human TACC proteins are involved in regulating the interaction between centrosomes and microtubules, this could explain the proposed links between the TACC genes and cancer. In *Drosophila* embryos, decreasing the levels of D-TACC at the centrosome leads to failures in pronuclear fusion, nuclear migration, and chromosome segregation and ultimately to embryonic death. Conversely, increasing the levels of D-TACC also leads to chromosome segregation defects and to a significant reduction in embryonic viability (J.W.R. and K. Jeffers, unpublished work). Thus, perturbing the levels of the human TACC proteins could cause similar defects in human cells, and this could contribute to the large-scale genetic instability that is a common feature of many, if not all, cancers (29–35). This could also explain the apparent paradox of why the TACC proteins have been suggested to be transforming proteins (15, 16), whereas TACC2 has also been isolated as a tumor suppressor protein (17); in *Drosophila*, increasing or decreasing the levels of D-TACC leads to an increase in genetic instability.

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Interaction of the transforming acidic coiled-coil 1 (TACC1) protein with ch-TOG and GAS41/NuBI1 suggests multiple TACC1-containing protein complexes in human cells

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Dysregulation of the human transforming acidic coiled-coil (TACC) proteins is thought to be important in the evolution of breast cancer and multiple myeloma. However, the exact role of these proteins in the oncogenic process is currently unknown. Using the full-length TACC1 protein as bait to screen a human mammary epithelial cDNA library, we have identified two genes that are also amplified and overexpressed in tumours derived from different cellular origins. TACC1 interacts with the C-terminus of both the microtubule-associated colonic and hepatic

tumour overexpressed (ch-TOG) protein, and the oncogenic transcription factor glioma amplified sequence 41/NuMA binding protein 1 (GAS41/NuBI1; where NuMA stands for nuclear mitotic apparatus protein 1). This suggests that the TACC proteins can form multiple complexes, dysregulation of which may be an important step during tumorigenesis.

Key words: breast cancer, glioma, transcription factors, microtubules.

INTRODUCTION

Tumorigenesis is a multistep process involving many genes. The accumulation of genetic changes, such as structural chromosomal abnormalities, is often associated with the increase in the malignant potential of cancer cells. Thus, in order to understand the complex events leading to the development and progression of cancer, it is necessary to identify and characterize candidate genes that may be involved in both the initiation and progression of the disease. Recently, we have identified a novel family of evolutionarily conserved genes, named the transforming acidic coiled-coil (TACC) genes, which are characterized by the presence of a large coiled-coil motif (the TACC domain) located at the C-terminus of each family member [1,2].

Several pieces of evidence implicate the TACC family in oncogenic processes. First, the human TACC genes are located in regions of the genome that are associated with cancer: the amplification or rearrangement of the chromosomal regions containing the TACC1 and TACC2 genes is implicated in breast tumour progression [1,3], while the third family member, TACC3, is located within 200 kb of a translocation breakpoint associated with multiple myeloma [2]. Secondly, *in vitro* and *in vivo* studies indicate that the TACC proteins are intimately linked to the processes of cell growth and differentiation. TACC1 and TACC3 are expressed at high levels during embryogenesis and are then down-regulated in differentiated tissues [1,2,4]. However, both TACC1 and TACC3 are expressed at high levels in human cancer cell lines [1,2]. TACC1 has the properties of a classic oncogene, in that it can transform mouse fibroblasts and promote anchorage-independent growth, which are characteristics shown by metastatic cancer cells [1]. Thus TACC1 and TACC3 are likely to be involved in the processes that promote cell division prior to the formation of differentiated tissues. Unlike TACC1 and TACC3, expression of the second member of this family, TACC2, is widespread in the adult (I. H. Still, B. Lauffart and O.

Gangisetty, unpublished work); however, in a model for breast tumour progression, TACC2 is down-regulated as breast tumours become more malignant [5]. Furthermore, reintroduction of the partial TACC2 clone, anti-zuai-1 ('AZU-1'), into these breast tumour cells reduces the ability of these malignant cells to grow [5]. Thus TACC2 has the properties of a breast tumour suppressor gene. Interestingly, the transformation of mouse fibroblasts by the oncogene, Ha-Ras, leads to an increase in TACC2 mRNA levels, suggesting that induction of TACC2 may be important in oncogenic cell signalling events initiated by other known oncogenes [6].

To begin to understand the functional role of the TACC proteins, we previously investigated the normal subcellular distribution of the human TACC-1, -2 and -3 proteins [7]. During interphase, the TACC proteins are distributed throughout the cell, with TACC1 and TACC3 showing a preferential accumulation in the nuclei of cells examined. Interestingly, as predicted from the protein sequence, antibodies raised against the human TACC proteins also stain the mitotic spindle and the centrosomes in mitotic HeLa and primary fibroblast cells [7]. Recently, the TACC domain of the *Drosophila* TACC (D-TACC) protein was shown to bind to msp's ('mini spindles'), the *Drosophila* homologue of the human colonic and hepatic tumour overexpressed (ch-TOG) protein [8,9]. This has led to the proposal that this interaction plays a role in the stabilization of centrosomal microtubules [8,9]. Murine TACC3 has also been shown to interact with the members of the arylhydrocarbon nuclear translocator (ARNT) family, and is also known as ARNT interacting protein (AINT) [4]. These transcription factors play critical roles in embryogenesis, cellular responses to chemical carcinogens and tumour progression/metastasis. Murine Tacc3 interacts with ARNT1 and can up-regulate ARNT-mediated responses to hypoxia and the carcinogen dioxin [4]. Thus it is still unclear whether the sole role of the TACC proteins is in the organization of the microtubule network by acting as

Abbreviations used: ARNT, arylhydrocarbon nuclear translocator; AINT, ARNT interacting protein; ch-TOG, colonic and hepatic tumour overexpressed; DAPI, 4,6-diamidino-2-phenylindole; NuMA, nuclear mitotic apparatus protein 1; GAS41/NuBI1, glioma amplified sequence 41/NuMA binding protein 1; GFP, green fluorescent protein; TACC, transforming acidic coiled-coil; D-TACC, *Drosophila* TACC; TACIP, TACC-interacting protein.

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adaptor molecules between non-microtubule proteins and microtubule-associated proteins, or whether the TACC proteins perform additional functions distinct from their association with microtubules. The identification of additional TACC-interacting proteins (TACIPs) is therefore likely to shed further light on the function of these proteins in normal and tumour cells.

We now present results of screening a yeast two-hybrid library for TACIPs. First, we demonstrate that TACC1 binds to the C-terminal section of the microtubule-associated human ch-TOG protein. In addition, we show that TACC1 interacts with the oncogenic transcription factor glioma amplified sequence 41/NuMA binding protein 1 (GAS41/NuB1; where NuMA stands for nuclear mitotic apparatus protein 1). Both ch-TOG and GAS41/NuB1 are amplified and up-regulated in cancers of different cellular origins. This suggests that the TACC proteins form multiple complexes, dysregulation of which is an important step during tumorigenesis.

MATERIALS AND METHODS

Yeast two-hybrid analysis

The Matchmaker yeast two-hybrid system and human mammary epithelial cDNA library were obtained from ClonTech Laboratories (Palo Alto, CA, U.S.A.). To construct the bait plasmid, pASTACC1, the TACC1 initiator start methionine was first replaced by a *SalI* site using PCR directed site-directed mutagenesis. Subsequently, the TACC1 open reading frame and approx. 200 bp of the 3' untranslated region was cloned into the GAL4 DNA-binding domain vector pAS2.1 (ClonTech Laboratories). This construct was transformed into yeast strain CG1945 using the lithium acetate method, and lack of autoactivation or non-specific interactions between the bait TACC1 plasmid and the GAL4 activation domain and lamin C negative control proteins indicated that the entire open reading frame could be used for screening. Library screening was carried out by the sequential transformation method according to the manufacturer's instructions. Clones that activated the *His3* and *LacZ* reporter genes only in the presence of the pASTACC1 plasmid were considered positive. The pACT2 plasmids containing cDNAs encoding potential TACIPs were isolated using the glass bead-phenol/chloroform method (Clontech Laboratories; Yeast Protocols Handbook PT3024-1) and transformed into chemically competent *Escherichia coli* cells (strain DH5- α ; Invitrogen, Carlsbad, CA, U.S.A.). Clones were sequenced using an ABI sequencer, courtesy of the Cleveland Clinic Foundation DNA sequencing core facility.

Elucidation of protein binding domains of GAS41 and TACC1

Subclones of TACC1 and GAS41 were generated by PCR and cloned into pAS2.1 and pACT2 respectively (primer sequences are available on request). Clones were sequenced prior to transformation into yeast. pASTACC1 and subclones of TACC1 were transformed into the yeast strain Y187 (ClonTech Laboratories), and pACT2-GAS41 subclones were transformed into CG1945. Expression of each construct was confirmed by Western-blot analysis using a monoclonal antibody raised against the GAL4 domain of the fusion protein (results not shown). Interactions between bait and target plasmids were examined using mating assays, according to the manufacturer's protocols.

Generation of stable cell lines expressing green fluorescent protein (GFP)-tagged GAS41

GAS41 cDNA was amplified from human brain cDNA using gene specific primers GAS41IMET (5'-GGACAATTGGGTC-

AAGAGAATG-3') and GAS41R2 (5'-TTTCTCGAGCTTAC-TACCAAG-3'). The amplified product was cloned into pCR2.1 (Invitrogen), and sequenced to confirm validity. The GAS41 insert was then isolated using *MfeI* and *XhoI* (these sites are shown in bold in the primer sequences) and cloned into the *EcoRI/SalI* site of the pEGFPC3 vector (ClonTech Laboratories). A stable cell line expressing EGGAS41 was then generated by transfecting pEGGAS41 into HEK-293 cells and the breast cancer cell line MDA-MB-468, as described previously [1]. Stable GAS41-resistant clones were examined by fluorescence microscopy, and EGGAS41 protein was detected by Western-blot analysis.

Immunological reagents

An immunogenic peptide, corresponding to residues 220–232 (PELVPSRRSKLRK; single-letter amino acid notation) of TACC1 was selected and generated by the multiple antigen peptide-synthesis method (Cleveland Clinic Foundation Biotechnology Core). This peptide was then used to produce a polyclonal antiserum to TACC1 in rabbits (performed by Rockland Immunochemicals, Boyertown, PA, U.S.A.). To confirm specificity, the antiserum was tested using immunohistochemical assays, and immunoprecipitation of GFP-tagged TACC1 with an antibody raised against GFP (#8372-2; ClonTech Laboratories), followed by Western-blot analysis, using the termination bleed antiserum (1:3000 dilution; results not shown). In native HEK-293 and MDA-MB-468 cells, the TACC1 antibody detects a protein of 110 kDa. Secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

Co-immunoprecipitations and Western-blot analysis

HEK-293 cells were washed with ice-cold PBS, prior to scraping off in RIPA buffer [50 mM Tris/HCl (pH 7.2, at 25 °C), 150 mM NaCl, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS and a cocktail of protease inhibitors]. The cell extract was passed several times through a 21-gauge needle and centrifuged at 3500 *g* for 15 min at 4 °C. Following centrifugation, the supernatant was removed and 200–500 μ g of extract was incubated with primary antibody (2 μ g) for 1 h at 4 °C. Anti-rabbit IgG agarose conjugate (#A8914; Sigma, St Louis, MO, U.S.A.) was then added and immunoprecipitation was allowed to proceed for an additional 1 h at 4 °C. Immune complexes were pelleted by centrifugation (1000 *g* for 5 min at 4 °C) and washed three times with RIPA buffer, and immunoprecipitated proteins were eluted by boiling with 2 \times Laemmli buffer [125 mM Tris/HCl (pH 6.8, at 25 °C), 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol and 0.004% (w/v) Bromophenol Blue]. Cell lysates and eluted complexes were separated by SDS/PAGE (8% gels) and transferred on to Immobilon membranes (Millipore, Marlborough, MA, U.S.A.). After blocking with TBS [10 mM Tris/HCl (pH 8, at 25 °C)/150 mM NaCl]/Tween 20 (0.02%, v/v) containing 5% (w/v) skimmed milk powder, the membrane was incubated with TACC-specific antisera in blocking buffer for 16 h at 4 °C. The membranes were washed with TBS/Tween 20 (0.02%, v/v) and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody. After washing, protein was visualized with the ECL[®] Plus detection system (Amersham Biosciences, Piscataway, NJ, U.S.A.).

Indirect immunofluorescence

HEK-293 and MDA-MB-468 cells were cultured on glass coverslips overnight in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum. Cells were fixed with 2%

(v/v) formaldehyde for 15 min, permeabilized with 0.2% Triton X-100 and then blocked with 10% (v/v) normal rabbit serum prior to incubation with the anti-TACC1 serum at 1:100 dilution for 1 h. The primary antibody was detected with a rhodamine-conjugated anti-rabbit antibody (Santa Cruz Biotechnology), and nuclei were counter-stained with 4,6-diamidino-2-phenylindole (DAPI). Cells were examined at 40 \times magnification. All procedures were carried out in the Roswell Park Cancer Institute Cell Analysis Facility.

RESULTS

Identification of TACIPs

To identify proteins that interact with TACC1, we fused the full-length TACC1 open reading frame to the GAL4 DNA-binding domain of the pAS2.1 vector. After confirming that this construct was unable to autoactivate the reporter genes in the CG1945 yeast host strain, this bait protein was used to screen an adult mammary epithelial cDNA library (ClonTech Laboratories). Approx. 10⁶ transformants were plated and selected on His⁻ selective medium containing 10 mM 3-aminotriazole. Of the 69 His⁺ clones originally isolated, 38 proved positive when assayed for β -galactosidase activity using the colony lift assay. Subsequent isolation and sequence analysis revealed that four of these clones corresponded to the C-terminal 529 amino acids of a previously identified TACC binding protein, the human ch-TOG protein (Figure 1A), and that five corresponded to the C-terminal region of the putative oncogenic transcription factor GAS41/NuB11 (Figure 1B).

Mapping of binding domains on GAS41/NuB11 and TACC1

TACIP38, the smallest of the GAS41/NuB11 cDNA clones identified, corresponded to the C-terminal 109 amino acids of GAS41/NuB11 (Figure 1B). The final 60 amino acids of this region is predicted to form an α -helical region, which has recently been shown to bind to the nuclear matrix protein and mitotic spindle component, NuMA [10]. To determine whether the TACC1 binding site on GAS41/NuB11 overlapped with that for NuMA, we generated an additional smaller construct containing the C-terminal amino acids 168–227. This construct activated the *His3* and *LacZ* reporter genes in the diploid strain only in the presence of the pASTACC1 construct, demonstrating that the TACC1 binding occurs within the predicted NuMA binding domain (Figure 1B). This suggests that TACC1 and NuMA could compete for binding to GAS41/NuB11.

To date, the C-terminal TACC domain has been shown to be important for the interaction of the TACC proteins with components of the microtubule network [8,9], and the ARNT transcription factors [4]. To determine whether the TACC domain also constituted the GAS41/NuB11 interacting domain, we next sought to assay the ability of GAS41 to bind to a series of smaller TACC1 constructs. These constructs were expressed as GAL4 DNA-binding domain constructs in yeast strain Y187. TACIP38 (in yeast strain CG1945) was then mated to each construct and diploids were assayed for specific interaction with each region of TACC1. Figure 2(A) demonstrates that activation of the reporter genes was only observed with pASTACC1 constructs containing the region between amino acids 206–427. The region between amino acids 263–446 contains three highly acidic imperfect repeats of 33 amino acids, which, based upon their amino acid composition, we have termed SDP repeats (Figure 2B). Hence, the region spanning these repeats appears to be the major determinant for TACC1 binding to GAS41.

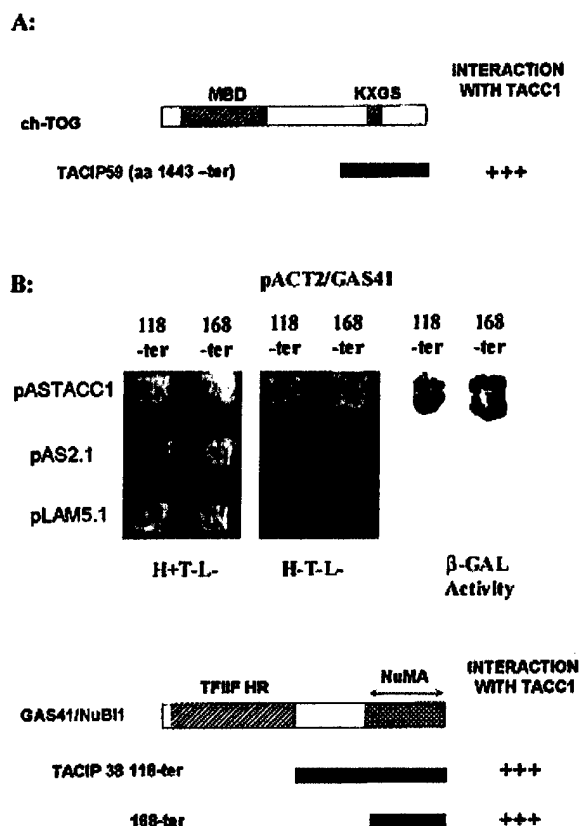


Figure 1 *In vitro* interactions between TACC1 and potential TACIPs defined by yeast two-hybrid analysis

(A) Yeast two-hybrid screening with the full-length TACC1 open reading frame identified four clones corresponding to amino acids 1443–C-terminus (-ter) of ch-TOG. This region contains the KXGS motif responsible for binding tubulin dimers, and is distinct from the microtubule binding domain (MBD). (B) Two GAL4-activation domain–GAS41 constructs, TACIP38 and GAS41 168-ter, were assayed for their ability to interact with the full-length TACC1 open reading frame (pASTACC1) using the yeast two-hybrid system. In mating assays, both constructs were able to activate the *His3* reporter, permitting growth on medium lacking histidine, and the *LacZ* gene [positive for β -galactosidase (β -GAL) activity], only in the presence of the TACC1 open reading frame. Neither construct activated the reporter genes in the presence of the pAS2.1 vector alone or the non-specific control pLAM5.1, expressing lamin C. The TACC1 binding site of GAS41/NuB11 is located in the C-terminal α -helical domain, overlapping the NuMA binding site. The general transcription factor IIIF homology region (TFIIIF HR) occupies the N-terminal 117 amino acids of GAS41/NuB11. H, histidine; T, threonine; L, leucine.

In vivo interaction between GAS41/NuB11 and TACC1

To determine whether TACC1 and GAS41 associate *in vivo*, we first stably transfected HEK-293 cells with a cDNA encoding the GAS41/NuB11 protein fused to the C-terminus of GFP (EGGAS41). Western-blot analysis of the stable cell line EGGAS41 confirmed that the GFP fusion product was correctly expressed (results not shown), and this clone was selected for further analysis. TACC1 specifically co-immunoprecipitated with the EGGAS41 fusion protein, using an antibody raised against the GFP moiety (Figure 3). This interaction was dependent on the presence of the GAS41 moiety, as GFP alone failed to co-immunoprecipitate with TACC1. Thus GAS41/NuB11 and TACC1 are found in the same complex *in vivo*.

To confirm further that GAS41/NuB11 and TACC proteins could physically interact in the intact cell, we determined whether GAS41/NuB11 co-localized with endogenous TACC1 *in vivo*.

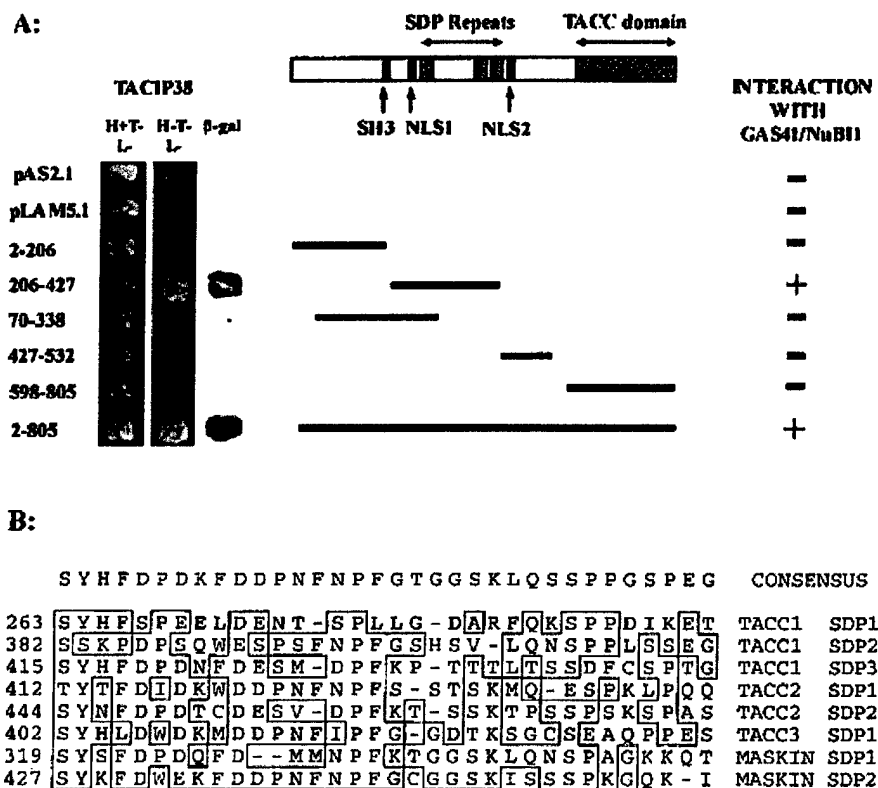


Figure 2 The GAS41 binding domain of TACC1 is located in the region containing the conserved SDP repeats

(A) To define further the binding domain for GAS41 on TACC1, a series of smaller TACC constructs were constructed and expressed as GAL4 DNA-binding domain constructs in yeast strain Y187. TACIP38 (in yeast strain CG1945) was mated to each construct and assayed for specific interaction with each region of TACC1. Interaction was only obtained with constructs containing amino acids 206–427, which contains the SDP repeats, and nuclear localization signal (NLS)1. SH3, Src homology 3. H, histidine; T, threonine; L, leucine; β -gal, β -galactosidase. (B) Sequence comparison of the SDP repeats from the TACC protein family. Each human TACC protein has a different number of these repeats. The *Xenopus* maskin protein also contains two SDP repeats, suggesting an evolutionary conserved function for the SDP repeat.

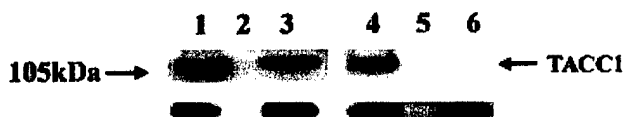


Figure 3 *In vivo* interactions between GAS41 and TACC1

Stable HEK-293 transfectants expressing either GFP fused to GAS41 (EGGASB1) or GFP alone (EGFP/HEK-293) were immunoprecipitated with either anti-GFP or rabbit IgG and immunoblotted with anti-TACC1 antibody. Due to its structure and acidic nature, TACC1 migrates at 110 kDa (compared with the predicted molecular mass of 88 kDa) in EGGASB1 and EGFP/HEK-293 cells (lanes 1 and 4 respectively) and is specifically immunoprecipitated from EGGASB1 cells by the anti-GFP antibody (lane 3), but not control IgG (lane 2). No interaction between GFP and TACC1 was detected in immunoprecipitates of EGFP/HEK-293 expressing GFP alone (lane 6). Lane 5 represents EGFP/HEK-293 immunoprecipitated with control IgG. Bottom panels confirm that similar amounts of GFP–GAS41 (53 kDa) and GFP (27 kDa) are immunoprecipitated by the anti-GFP antibody in lanes 3 and 6.

Stable transfection of the EGGAS41 plasmid into HEK-293 cells, and the breast cancer cell line MDA-MB-468 resulted in an accumulation of GFP staining in the nucleoplasm, but not the nucleoli of these cells (Figure 4). Indirect immunofluorescence microscopy using the TACC1 antibody revealed a similar staining pattern to the EGGAS41 fusion protein, together with some low-level diffuse staining in the cytoplasm. The nuclear accumulation

of TACC1 in these transfected cell lines was not a side effect of the overexpression of the GFP fusion protein, as no difference in TACC1 localization was noted in HEK-293 and MDA-MB-468 cells transfected with GFP vector alone (Figure 4) or untransfected cells (results not shown). Therefore the primary site of interaction between GAS41 and TACC1 is likely to be the nuclei of interphase cells.

DISCUSSION

The evolutionarily conserved TACC family is comprised of proteins found in mammals [1,2,4], *Xenopus* [11] and *Drosophila* [12]. Originally, based upon their protein sequence, the human TACC proteins were predicted to play a role in nuclear scaffolding and/or mitotic spindle assembly [1]. These potential roles have been partly supported from studies of their subcellular localization [7]; however, further clues as to their functional roles will only come from the identification of potential TACIPs. We have now begun a systematic attempt to isolate these proteins by yeast two-hybrid analysis. Using this method, we have now demonstrated that TACC1 interacts with the microtubule-associated protein ch-TOG, and the oncogenic transcription factor/NuMA-binding protein GAS41/NuB11. Expression analysis has revealed that TACC1 and TACC3 are expressed in several tissues prior to the onset of differentiation [1,2,4]. However, both TACC1 and

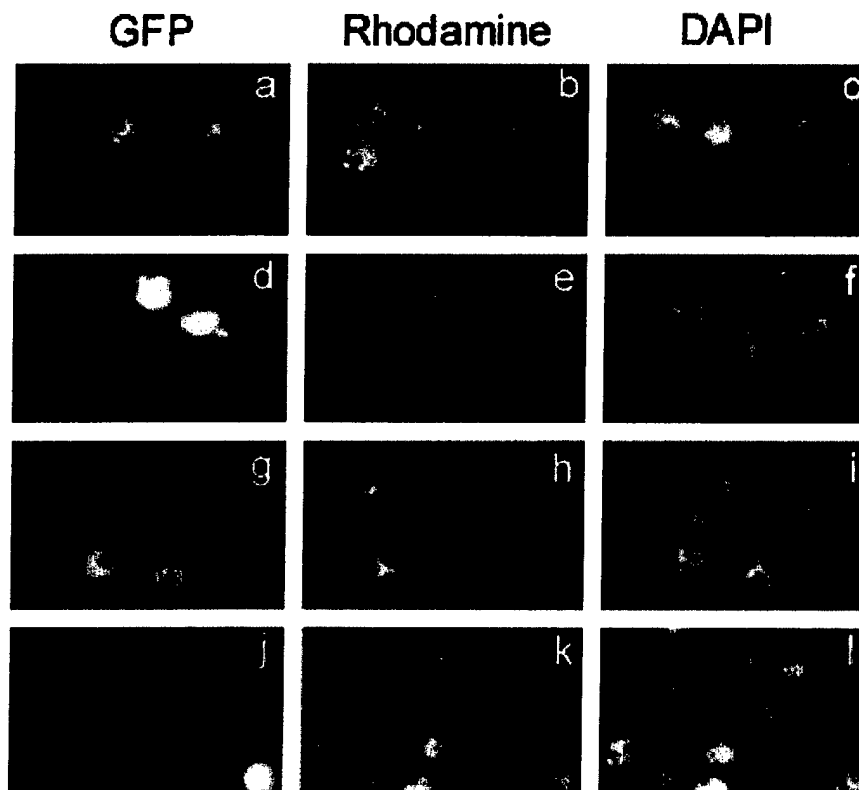


Figure 4 Co-localization of EGGAS41 and native TACC1 in HEK-293 and MDA-MB-468 cells during interphase

Full-length GAS41 fused to GFP is predominantly expressed in the nucleoplasm of stably transfected HEK-293 and MDA-MB-468 cells (a and g respectively). Indirect immunofluorescence using the TACC1 antibody and a rhodamine-labelled secondary antibody also shows that TACC1 is predominantly localized to the nuclei of the same cells (b and h). Nuclear accumulation of TACC1 is independent of the expression of a GFP fusion protein, since HEK-293 and MDA-MB-468 cells expressing GFP alone (in the cytoplasm and nucleus of transfected cells; d and j), also show predominantly nuclear expression of TACC1 (e and k respectively). Nuclei were counterstained with DAPI (c, f, i and l).

TACC3 are expressed at high levels in human cancer cell lines. Similarly, ch-TOG is overexpressed in colonic and hepatic tumours, relative to normal tissues [13], and GAS41/NuBII is both amplified and overexpressed at early stages of glioblastoma tumorigenesis [14]. Conversely, TACC2 has been shown to be down-regulated in a model for breast tumour progression [5]. Therefore regulation of the interaction between TACC, ch-TOG and/or GAS41/NuBII proteins may be critically important to the control of division of tumour cells derived from different origins.

During interphase, the human TACC proteins are found at low levels within the cytoplasm, with an increased accumulation of TACC1 and TACC3 in the nuclei of most cells within the cell population (Figure 4) [7]. Upon entering mitosis, the TACC proteins begin to associate with the centrosome and the mitotic spindle, although the interaction between TACC proteins and microtubules appears to be indirect, requiring the presence of another protein [7]. The ch-TOG protein fulfils this role in that it is able to interact directly with microtubules [15]. Interestingly, the clones that we identified corresponded to the C-terminal 529 amino acids of ch-TOG. This region contains the tubulin dimer-binding domain, required for microtubule nucleation [15]. Thus, by binding to the C-terminus of ch-TOG, TACC1 could either compete with, or stabilize, the binding of the tubulin dimer to ch-TOG. Alternatively, through its interaction with ch-TOG, TACC1 could bring regulatory proteins into the vicinity of the

growing microtubule. Recently, the D-TACC protein was shown to bind to the *Drosophila* ch-TOG homologue, msps [8,9]. Furthermore, analysis of the msps and D-TACC proteins suggested that, through this interaction, D-TACC could be involved in anchoring and stabilizing microtubules to the centrosome [8,9]. This model has also been proposed for one of the functions of human TACC proteins associated with microtubules [9].

The second TACIP that we identified was the putative transcription factor GAS41/NuBII. This protein binds *in vitro* to the nuclear matrix component NuMA, which is itself important in the assembly of the mitotic spindle. Similarly to TACC proteins, NuMA forms large oligomeric structures when overexpressed in human cells [7,16]. However, we have not been able to co-immunoprecipitate TACC1 with NuMA (results not shown), and NuMA polymers do not contain a detectable level of TACC1 protein [7], indicating that NuMA and TACC1 are found in distinct complexes. As NuMA only interacts weakly with GAS41/NuBII in mammalian cells [10], TACC1 may be the preferential GAS41/NuBII binding partner in interphase cells.

GAS41/NuBII is a highly conserved protein with homologues in vertebrates, invertebrates, plants and fungi [10]. The degree of homology between the human and *Drosophila* proteins (61% identity and 70% overall similarity) raises the possibility that D-TACC may also bind the *Drosophila* GAS41/NuBII counterpart. However, the GAS41/NuBII binding site of TACC1 is not conserved in the D-TACC protein, indicating that other serine/

acidic-rich regions may act as *Drosophila* GAS41/NuBII binding sites. Of evolutionary importance is the fact that, to date, no TACC homologue has been detected in either plants or yeast, suggesting that the TACC-GAS41/NuBII interaction plays a unique role in animal biology.

Sequence analysis has indicated that the GAS41/NuBII protein is related to AF-9 and ENL ('acute lymphoblastic leukaemia 1 fused gene from chromosome 9' and 'eleven-nineteen leukaemia gene'), which are putative transcription factors rearranged in some acute leukaemias [14]. The region of homology between these proteins is related to the general transcription initiation factor Tfg3/TAF30/Anclp ('transcription factor G 30 kDa subunit/TATA-binding protein associated factor 30 kDa subunit/actin non-complementing protein 1') [17]. This protein is an essential component for basal transcription and plays an important role in mediating interactions between sequence-specific transcription factors and the RNA polymerase II transcriptional machinery [18]. This suggests that GAS41/NuBII is involved in regulating gene transcription through a direct interaction with the basal transcription initiation complex. The predominantly nuclear localization of GAS41/NuBII also partially supports this idea (Figure 4) [10,19]. However, to date, GAS41/NuBII has not been shown to bind directly to a known transcription factor, suggesting that an accessory protein may be required for GAS41/NuBII to bind to DNA-sequence-specific transcription factors.

We have demonstrated that native TACC1 and TACC3 show enhanced accumulation in the nuclei of cells in culture (Figure 4), [7]. Nuclear accumulation of TACC2 also increases in human microvascular endothelial cells in response to treatment with erythropoietin [20]. This suggests that TACC proteins may play a role in signal transduction to the nucleus in response to certain cytokines. The murine Tacc3 protein, AINT, has recently been shown to interact via the TACC domain with the ARNT transcription factors [4]. Thus it is tempting to speculate that TACC proteins may be involved in the final stages of signal transduction in the nucleus, providing a structural link enhancing the binding of DNA-sequence-specific transcription factors, such as ARNT, to a GAS41/NuBII-containing basic transcription factor complex in the nucleus. This hypothesis is further supported by the observation that overexpression of Tacc3 can enhance the ARNT-mediated hypoxic induction of the erythropoietin promoter, and can also increase the activation of a xenobiotic response element-luciferase reporter by dioxin [4]. Dysregulation of TACC and GAS41/NuBII proteins may therefore contribute to tumorigenesis by altering the transcriptional response to cell signalling pathways.

In conclusion, it appears that the TACC proteins can form multiple different protein complexes in the cell. In the cytoplasm, TACC proteins appear to act as scaffolding/bridging proteins important for centrosomal function. The TACC proteins may perform a similar function in the nuclear matrix, by acting as scaffolding or bridging proteins between transcription factors and basal transcription initiation complexes. This potential transcriptional regulatory role of the TACC proteins may be particularly important in tumorigenesis, and now represents a new avenue of TACC research that needs to be addressed.

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