UNIVERSITY OF CALIFORNIA, SAN DIEGO

Proteins that Interact with *Arabidopsis* TANGLED

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Carolyn Rasmussen is a co-author of Figure 5 (Localization of RAB GAP :: YFP during telophase) and Figure 6 (Localization of YFP :: PHOX2 during telophase) and has given me the permission to use these two Figures in this Thesis. These two Figures have not been published, have not been submitted for publication, and are not currently being prepared for publication.

ABSTRACT OF THE THESIS

Proteins that Interact with *Arabidopsis* TANGLED

by

Tianying Su

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Laurie G. Smith, Chair

TANGLED is a plant-specific protein that plays an important role in cell division plane orientation. So far, most of the research on TANGLED has focused on the requirements for its localization in dividing cells. However, it is still unknown how these requirements work together to promote TANGLED localization. Furthermore,

the mechanisms by which TANGLED functions in cell division plane orientation are unknown. There are most likely proteins that interact with TANGLED to help TANGLED localize and function. So far, the only protein known to interact with *Arabidopsis* TANGLED is PHRAGMOPLAST ORIENTING KINESIN 1 (POK1). Using yeast-two-hybrid assay, I screened through a cDNA library of *Arabidopsis thaliana* and identified eight proteins that are new promising candidates for bona fide interaction with TANGLED. Also using yeast-two-hybrid, I found that the first 132 amino acids of TANGLED are necessary and sufficient for its interaction with three of these eight proteins. For two of the eight proteins, localization experiments involving fusion with yellow fluorescent protein revealed that they localize to the cell plate in dividing *Arabidopsis thaliana* cells.

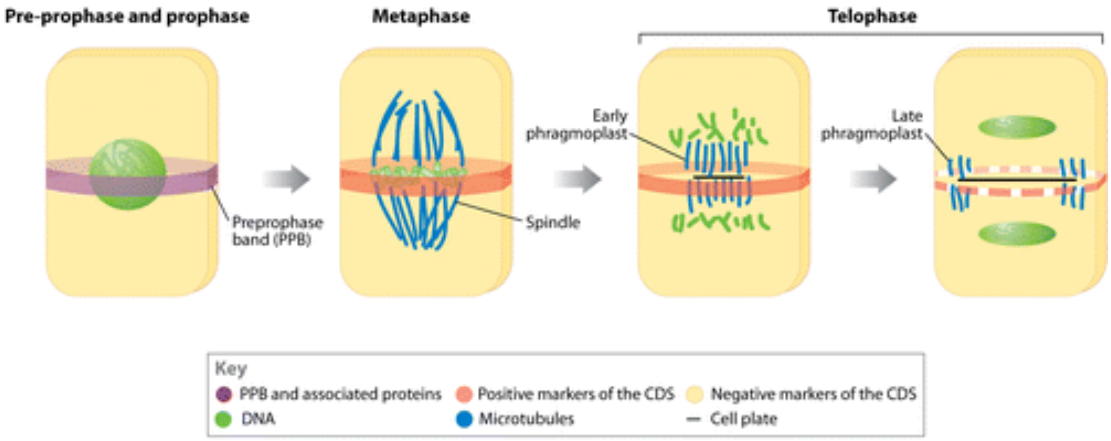
INTRODUCTION

Spatial regulation of cell division in plants

During plant cell division, several structures unique to plants form, making plant cell division different from cell division in other organisms. During Gap 2 phase of the cell cycle, a structure called the preprophase band forms (Wright and Smith, 2008). The preprophase band consists of microtubules and F-actin (Palewitz, 1987). It is shaped like a ring and it is found at the cortex of the cells (Pickett-Heaps and Northcote, 1966). The preprophase band marks down the site of the future cell division (Pickett-Heaps and Northcote, 1966). The plant cell then continues through prophase. At the transition between prophase and metaphase, the preprophase band disappears (Pickett-Heaps and Northcote, 1966). During metaphase, the mitotic spindle forms and distributes the chromosomes as in an animal cell (Pickett-Heaps and Northcote, 1966).

Then, during anaphase, a structure called the phragmoplast forms (Staehelin and Hepler, 1996). The phragmoplast consists of two equally-sized parallel cylindrical clusters of organized microtubules, microfilaments, Golgi-derived vesicles, and endoplasmic reticulum (Staehelin and Hepler, 1996). At the beginning of telophase, each of these two cylindrical clusters of the phragmoplast occupies the middle portion of a cross section of the cell (Staehelin and Hepler, 1996; Rasmussen et al., 2011b). During telophase and cytokinesis, the Golgi-derived vesicles in the phragmoplast fuse together to construct the cell plate (the structure that separates two daughter cells), (Samuels et al., 1995). These vesicles fuse at the gap that's sandwiched between the

two clusters of the phragmoplast (Samuels et al., 1995). Therefore, the phragmoplast acts as a guide for building the cell plate. The formation of the cell plate begins at the center of the cell and continues outwards across the cell (Samuels et al., 1995). As the formation of each part of the cell plate reaches completion, the phragmoplast that sandwiched that part of the cell plate disappears (Samuels et al., 1995). New phragmoplast gets assembled at the edge of maturing cell plate and the process of Golgi vesicle directed formation of the cell plate continues until cell plate reaches the cortex of the cell (Rasmussen et al., 2011b). At the cell cortex, the phragmoplast guides the cell plate to the exact site of the long-gone preprophase band (Gunning, 1982). This site is called the cortical division site. Figure 1 shows the various cytoskeletal structures formed during cell division in plants.



Rasmussen CG, et al. 2011.
Annu. Rev. Plant Biol. 62:387–409

Figure 1. Spatial regulation of cell division in plants. This figure shows the locations of various cytoskeletal structures, such as the preprophase band and phragmoplast, during cell division. This is Figure 1 from the Rasmussen et al 2011b review.

There is a very intriguing aspect of plant cell division. Since the preprophase band (the marker for site of cell division) disappears during prophase, how does the plant cell then know where to divide after prophase? How does the phragmoplast know where to construct and attach the cell plate after the preprophase band disappears? There seems to be a missing link between the preprophase band and the phragmoplast.

There are several proteins that localize to the site of the preprophase band after the preprophase band disappears. RanGAP1 co-localizes with the preprophase band and remains at the cortical division site throughout mitosis and cytokinesis; it is also localized at the cell plate (Xu et al., 2008). RNA interference of RanGAP1 in *Arabidopsis* causes incomplete and misoriented cell walls (Xu et al., 2008). However, it is still not clear how exactly RanGAP1 functions in cell division plane orientation. DCD1 and ADD1 are nearly identical maize proteins and their *Arabidopsis* homolog is FASS, also called TON2 (Muller et al., 2009). DCD1 and ADD1 are the B subunits of PP2A phosphatase complexes (Muller et al., 2009). DCD1/ADD1 co-localizes with the preprophase band during prophase, and initially remains at the cortical division site after the preprophase band disappears, but then disappears from the cortical division site after metaphase (Wright et al., 2009). In maize, RNA interference of DCD1 and ADD1 causes misoriented cell divisions (Wright et al., 2009). Genetic studies reveal that DCD1/ADD1/TON2 is required for preprophase band formation; its role at the cortical division site after preprophase band formation remains to be determined (Wright et al., 2009; Camilleri et al., 2002). T-PLATE localizes to the cell plate, and also transiently localizes to the cortical division site as the cell plate attaches to the

cortex; it is necessary for the attachment of the cell plate to the cortex in BY2 tobacco cells (Van Damme et al., 2006). AIR9 is a microtubule-binding protein that co-localizes with the preprophase band during prophase (Buschmann et al., 2006). However, it disappears from the cortical division site when the preprophase band disappears, and throughout mitosis and cytokinesis it co-localizes with the mitotic spindle and the phragmoplast. Then, when the cell plate attaches to the cortical division site at the end of cytokinesis, AIR9 reappears again (Buschmann et al., 2006). Buschmann et al 2006 proposed that AIR9 is involved with ensuring the maturation of the cell plate that attaches to the cortical division site.

The absence of a protein at the cortical division site serves as a marker as well. The protein KCA1 localizes to the plasma membrane but it is depleted from the cortical division site throughout mitosis and cytokinesis (Vanstraelen et al., 2006). In BY2 tobacco cells, misplaced cell plates are found in cell divisions without this KCA1 depleted zone (Vanstraelen et al., 2006). Actin is also depleted from the cortical division site (Muller et al., 2009). Actin also appears in two peaks flanking the edges of the cortical division site (Muller et al., 2009). Figure 2 shows the localizations of the positive and negative markers of the cortical division site.

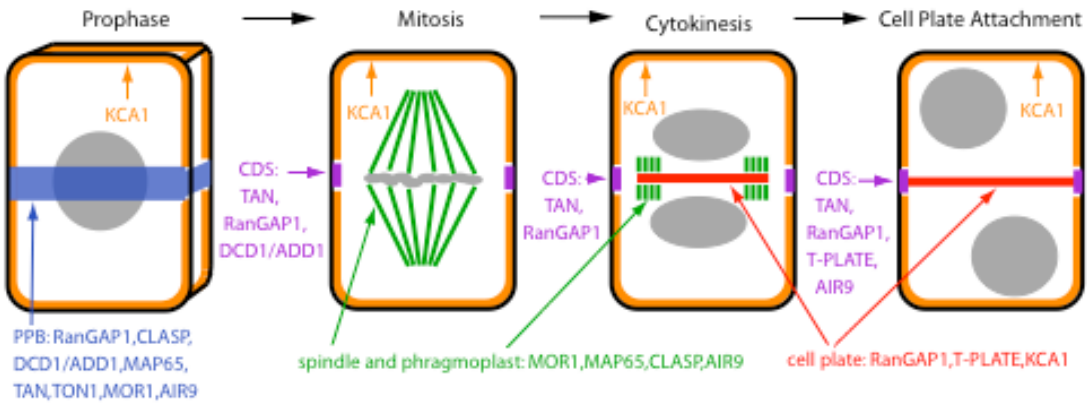


Figure 2. Positive and negative markers of cell division. This figure shows the localizations of the various proteins that mark the cortical division site in various stages of cell division. This is Figure 2 from the Muller et al 2009 review.

TANGLED

The protein TANGLED is another positive marker of the cortical division site. Maize *tangled* mutants have misoriented cell divisions, indicating that the phragmoplasts have failed to guide the cell plate to the proper division site established by the preprophase band (Cleary and Smith, 1998). *Arabidopsis thaliana tangled* mutants were also found to have misoriented phragmoplasts (Walker et al., 2007). In *Arabidopsis thaliana* cells undergoing preprophase, TANGLED has been found to co-localize with the preprophase band—it is localized as a ring on the cortex of the cell (Walker et al., 2007). However, even after the preprophase band disappears during prophase, TANGLED still stays at where the preprophase band used to be, continuing to mark the site of cell division until the complete formation of the cell plate (Walker et al., 2007). TANGLED disappears from the division site after the complete formation of the cell plate (Walker et al., 2007). TANGLED thus appears to be

important for marking the cortical division site after the disappearance of the preprophase band and orienting the expansion of the phragmoplast to the cell division plane established by the preprophase band.

TANGLED is a protein only found in plant cells (Rasmussen et al., 2011a). It is a strongly basic protein that binds microtubules (Smith et al., 2001). The expression of TANGLED is highest in dividing cells (Smith et al., 2001; Walker et al., 2007). It also has several phosphorylation sites (Sugiyama et al., 2008). In maize, two proteins have been found to interact with TAN1, the maize homolog of *Arabidopsis* TANGLED (Muller et al., 2006). These two proteins are most similar to the *Arabidopsis thaliana* proteins PHRAGMOPLAST ORIENTING KINESIN 1 (POK1) and PHRAGMOPLAST ORIENTING KINESIN 2 (POK2), (Muller et al., 2006). In *A. thaliana*, POK1 has been confirmed to interact with TANGLED by yeast-two-hybrid, and it interacts with amino acids #1-132 of TANGLED (Muller et al., 2006; Rasmussen et al., 2011a). *A. thaliana* double mutants lacking both PHRAGMOPLAST ORIENTING KINESIN 1 (POK1) and PHRAGMOPLAST ORIENTING KINESIN 2 (POK2) have misoriented cell plates, similar to *tangled* mutants (Muller et al., 2006).

So far, most of the published research on TANGLED focused on the mechanisms by which TANGLED localizes to the division site. Published experimental results revealed the specific requirements for TANGLED to localize to the division site in each stage of mitosis and cytokinesis. During preprophase and prophase, TANGLED localization to the division site requires microtubules, amino acids #126-229 of TANGLED, and the PP2A phosphatase activity (Walker et al.,

2007; Rasmussen et al., 2011a). During preprophase and prophase, POK1 and POK2 help to enhance the localization of TANGLED to the division site, but they're not necessary for it (Walker et al., 2007). During metaphase, the localization of TANGLED to the division site requires all amino acids of TANGLED (no single part of the protein has been identified yet as necessary and sufficient for localization during this stage), the successful formation of the preprophase band during preprophase, and PP2A phosphatase (Rasmussen et al., 2011a). Microtubules are not required for TANGLED localization to the division site during metaphase (Walker et al., 2007). From anaphase to cytokinesis, TANGLED localization to the division site requires amino acids #1-132 of TANGLED (which binds POK1), POK1, POK2, and the successful formation of the preprophase band during preprophase (Rasmussen et al., 2011a; Walker et al., 2007). Microtubules are not required for TANGLED to localize to the division site from anaphase to cytokinesis (Walker et al., 2007). The significant amount of published research summarized here shows that TANGLED localization to the cortical division site is a very dynamic process that requires several different molecular mechanisms (Rasmussen et al., 2011a).

My goals

Most of the research so far focused on the requirements for TANGLED localization. However, it is still unclear how exactly do all these different requirements for TANGLED localization work together to initiate and maintain TANGLED's localization to the division site. There are most likely other proteins involved in TANGLED localization. These proteins are yet to be identified. This leads

to the first goal of my research: to identify more proteins that interact with TANGLED with the purpose of obtaining a more comprehensive understanding of the molecular mechanisms by which TANGLED localizes to the division site during mitosis and cytokinesis. This goal is important because the successful localization of TANGLED to the division site is a prerequisite for TANGLED to perform its function of properly orienting phragmoplasts (Rasmussen et al., 2011a). Thus, having a more comprehensive understanding of TANGLED localization is a key step to understanding how do plant cells properly orient their cell division planes.

Since most of the research on TANGLED so far focused on TANGLED localization, it is still unknown how TANGLED interacts with the phragmoplast to orient the phragmoplast to the cell division plane established by the preprophase band. The molecular mechanisms of how TANGLED relays the preprophase band's message of "here is where the cell divides" to the phragmoplast are yet to be discovered. Since TANGLED and the phragmoplast don't co-localize until the end of cytokinesis when the phragmoplast has expanded to the cortex of the cell, there must be proteins that mediate that interaction between TANGLED and the phragmoplast/cell plate. There may be proteins that communicate the orientation of the phragmoplast/cell plate to TANGLED so that TANGLED can influence phragmoplast orientation. This leads to the second goal of my research: to identify more proteins that interact with TANGLED with the purpose of helping to uncover the mechanisms by which TANGLED guides phragmoplasts to the division site.

The achievement of both of these goals of my research requires the identification of proteins that interact with TANGLED. The method that I use to

identify proteins that interact with TANGLED is by yeast-two-hybrid assay (Fields and Song, 1989). Using yeast-two-hybrid, I screened an *Arabidopsis thaliana* cDNA library for proteins that interact with TANGLED.

MATERIALS AND METHODS

Screening through *Arabidopsis thaliana* cDNA library for positive transformants

First, YRG2 strain of competent *Saccharomyces cerevisiae* (yeast) already containing a pBD vector (the bait) recombined with TANGLED cDNA (pBD-TAN) was streaked onto solid CSM (Complete Synthetic Media) without tryptophan. The yeast was incubated at 28°C for three days. Then, this yeast was subcultured into liquid CSM without tryptophan and it is shaken at 30°C overnight. The pBD-TAN is described in Walker et al 2007. Next, recombinant pAD vectors containing a cDNA library of *Arabidopsis thaliana* was transformed into this yeast using the "Quick and easy yeast transformation using the LiAc/SS carrier DNA/PEG method" (Gietz and Schiestl, 2007). The recombinant pAD vectors containing the *Arabidopsis thaliana* cDNA library is the Horwitz and Ma Two-Hybrid cDNA Library, and it can be purchased from the Arabidopsis Biological Resource Center (ABRC) through the Arabidopsis Information Resource website (TAIR, http://www.arabidopsis.org). The mRNA used to generate this cDNA library was from the influorescence meristem, floral meristem, and floral buds of *Arabidopsis thaliana* at Stages 8 and 9 of development. The average size of insert was 1 kb.

Then, the yeast that was transformed with cDNA library was plated onto solid CSM lacking histidine, leucine, and tryptophan. The yeast was incubated at 28°C. On days five and seven, the largest colonies were streaked onto new plates of CSM lacking histidine, leucine, and tryptophan. The new plates were incubated at 28°C. At day two of incubation, the extent of growth of each inoculated yeast colony was

examined. The yeast colonies that had the most growth were the positive transformants. They were inoculated into liquid CSM lacking leucine and shaken at 28°C for two days before pAD plasmids were extracted from them.

pAD plasmid extraction from the positive transformants

The pAD plasmid was extracted from each positive transformant using the "A simple and highly efficient procedure for rescuing autonomous plasmids from yeast" (Robzyk and Kassir, 1992). The pAD plasmids extracted from the yeast were then transformed into Super Competent BP5α *E. coli* cells (Biopioneer) using the heat shock method. The transformed *E. coli* was plated on solid LB media with 50 µg/ml of ampicillin and then incubated at 37°C for 16-18 hours. One colony was chosen from each plate to represent one cDNA clone in pAD. The chosen colonies were inoculated into liquid LB media with 50 µg/ml of ampicillin and shaken at 37°C for 16-18 hours. Then, the pAD plasmids were extracted from grown cultures using a plasmid purification kit from one of these companies: Invitrogen, Fermentas, or Biopioneer.

Testing extracted cDNA clones in pAD for self-activators

In the first step of testing the extracted cDNA clones in pAD for self-activation, YRG2 strain of competent yeast already containing a recombinant pBD vector with TANGLED cDNA (pBD-TAN) was streaked onto plates of CSM without tryptophan. YRG2 strain of competent yeast already containing an empty (nothing inserted into the multiple cloning site) pBD vector was also streaked onto plates of CSM without tryptophan. The empty pBD vector is from Stratagene. Both types of yeast were

streaked in dense patches covering the entire plate. Both types of yeast were incubated at the same time for two to three days at 28°C. Then, each cDNA clone in pAD vector that was extracted from the *E. coli* was transformed into the grown yeast carrying the pBD-TAN and also into the grown yeast carrying an empty pBD using the "Quick and easy yeast transformation using the LiAc/SS carrier DNA/PEG method" (Gietz and Schiestl, 2007). For the positive control, pAD containing the cDNA of PHRAGMOPLAST ORIENTING KINESIN 1 (pAD-POK1) was transformed into the yeast containing the pBD-TAN. The pAD-POK1 was described in Muller et al 2006. For the negative control, pAD-POK1 was transformed into the yeast containing the empty pBD. The transformed yeast was then plated on solid CSM lacking leucine and tryptophan and incubated at 28°C for four to six days. Then, eight colonies were selected from a plate containing yeast transformed with a particular pAD and pBD-TAN; eight colonies were also selected from a plate of yeast transformed with the same cDNA clone in pAD and pBD empty. The colonies were streaked onto solid CSM lacking tryptophan, leucine, and histidine (CSM-L-T-H). The CSM-L-T-H plates were incubated at 28°C for seven days. Yeast growth on each of the CSM-L-T-H plates was observed each day and compared to the growth of the positive and negative controls for evaluation of self-activation. The cDNA clones in pAD that didn't self-activate were sent for sequencing (Retrogen). Sequences were analyzed using the BLAST tool on TAIR.

Testing promising candidates for interaction with different regions of TANGLED

To test the promising candidates for interaction with different parts of TANGLED, YRG2 competent yeast containing recombinant pAS vectors coding for the following regions of TANGLED (these plasmids have been used and described in Rasmussen et al 2011a): amino acids #1-132 (Region I), amino acids #126-473 (minus Region I), amino acids #126-229 (Region II), all amino acids except amino acids #132-222 (minus Region II), and amino acids #1-229 (Regions I and II) was streaked onto solid CSM lacking tryptophan (CSM-T). For the positive control, yeast containing pBD-TAN was streaked onto CSM-T. For the negative control, yeast containing empty pBD was streaked onto CSM-T. All types of yeast were streaked in dense patches. All types of yeast were incubated at the same time for two to three days at 28°C. The pAD vector containing the cDNA of the promising candidate was then transformed into each of these grown transgenic yeast using the "Quick and easy yeast transformation using the LiAc/SS carrier DNA/PEG method" (Gietz and Schiestl, 2007). The transformed yeast was plated onto CSM lacking leucine and tryptophan and incubated at 28°C for four to six days. Then, eight colonies were chosen from each plate and streaked onto CSM-L-T-H and incubated at 28°C for seven days. Yeast growth on each the CSM-L-T-H plate was observed each day and compared to the growth of the positive and negative controls for evaluation of interaction.

Localizations of RAB GAP and PHOX2

These experiments were done in collaboration with Carolyn Rasmussen, a postdoc from the Smith lab. A partial length cDNA coding for amino acids #550-778 of RAB GAP was cloned into pEZRK-LNY. A cDNA coding for full length PHOX2

in pENTR223 (from ABRC) was cloned into pEarleyGate-104 (from ABRC) using the Gateway cloning kit (Invitrogen). pEZRK-LNY is described in Walker et al 2007. pEarleyGate-104 is described in Earley et al 2006.

These constructs were transformed into competent *Agrobacterium tumefaciens* by electroporation. Then using floral dip method (Clough and Bent, 1998), the constructs were transformed from the *Agrobacterium* into flowering *Arabidopsis thaliana* with cerulean fluorescent protein (CFP) tagged alpha-tubulin (plants are from Viktor Kirik, Kirik et al., 2007). The transformed plants were grown at 20-22°C in a 16-hour light and 8-hour dark cycle. Seeds were harvested from these plants and were sterilized using 70% ethanol with 0.05% triton X 100. For RAB GAP, the seeds were plated on half-strength Murashige and Skoog media with 0.8% agar and 100 µg/ml of kanamycin. For PHOX2, the seeds were plated on half-strength Murashige and Skoog media with 0.8% agar, 1% sucrose, and 15 µg/ml of basta. The plates were stacked vertically to allow vertical growth of seedlings and the seeds were grown under constant light. The root tips of six to ten-day-old seedlings were observed with a spinning-disk confocal microscope. The CFP was excited with a 440 nm laser. Yellow fluorescent protein (YFP) was excited with a 514 nm laser. Z-stacks were taken at 1-2 µm intervals. The 3-D reconstructions of the z-stacks were performed using the program Metamorph and Image J. Photoshop was used to false color and merge the z-stacks. For PHOX2, the progeny of the transformants were visualized for CFP and YFP.

RESULTS

Screening through *Arabidopsis thaliana* cDNA library for positive transformants

To find proteins that interact with TANGLED, first I screened through an *Arabidopsis thaliana* cDNA library using the yeast-two-hybrid assay. The bait is the pBD vector with the DNA binding domain of yeast GAL4 fused to full length TANGLED cDNA (pBD-TAN). The prey is the pAD vector with the activating domain of yeast GAL4 fused to *Arabidopsis thaliana* cDNAs (pAD-cDNAs). The bait is screened against the prey. If TANGLED and the protein coded by a cDNA clone interact, then the production of histidine is activated, and thus the yeast will form a colony on a media lacking tryptophan (selective marker for pBD), leucine (selective marker for pAD), and histidine. I transformed the pAD-cDNA library into yeast that already contained the pBD-TAN and plated the transformants on media without tryptophan, leucine, and histidine. From the yeast colonies that grew on this media, I isolated the 224 largest colonies, and I called these colonies the positive transformants.

Identification of non-self-activators

Yeast's ability to produce histidine may also be caused by factors other than the interaction between TANGLED and the protein coded by the cDNA clone. This phenomenon is called self-activation. To ensure that the growth of yeast on media lacking tryptophan, leucine, and histidine is due to protein-protein interaction rather than self-activation, the pAD containing the cDNA clone from each positive transformant was extracted and then tested for self-activation. For the self-activation

test, the pAD plasmid containing the cDNA clone was transformed into yeast containing pBD recombined with TANGLED cDNA (pBD-TAN) and also into yeast containing the empty (nothing in multiple cloning site) pBD. If the protein coded by the cDNA clone is a self-activator, then the yeast containing this cDNA clone in pAD and the pBD-TAN will grow on media lacking leucine, tryptophan, and histidine (CSM-L-T-H), and the yeast containing this cDNA clone in pAD and an empty pBD vector will grow on CSM-L-T-H as well. If the protein coded by the cDNA clone is not a self-activator, then the yeast containing this cDNA clone in pAD will grow on CSM-L-T-H only when co-transformed with the pBD-TAN. An example of a protein that truly interacts with TANGLED and is not a self-activator is PHRAGMOPLAST ORIENTING KINESIN 1 (POK1). POK1 was identified by Muller et al 2006 as an interactor of TANGLED. Therefore, for the positive control (for determining if the yeast grew) of the self-activator test, I used yeast containing pBD-TAN and POK1 cDNA in pAD (pAD-POK1); for the negative control (for determining if the yeast failed to grow) of the self-activator test, I used yeast containing empty pBD and pAD-POK1. Figure 3 shows the positive and negative controls.

I successfully extracted pAD plasmids from 220 out of the 224 positive transformants and performed the self-activator test 219 of these. I also performed the self-activator test on 24 pAD plasmids that Brian Sun (a former lab member) extracted from the positive transformants in another yeast-two-hybrid screen for TANGLED interactors. Thus, I performed the self-activator test on a total of 243 cDNA clones. Out of these, I found that 30 cDNA clones were not self-activators. Figure 4 shows an example of such a non-self-activator. Table 1 shows the identities of the 30 cDNA

clones that code for the proteins that interact with TANGLED in yeast-two-hybrid. Because some of these cDNA clones are duplicates, a total of 17 proteins that interact with TANGLED are identified from the 30 cDNA clones (Table 1).

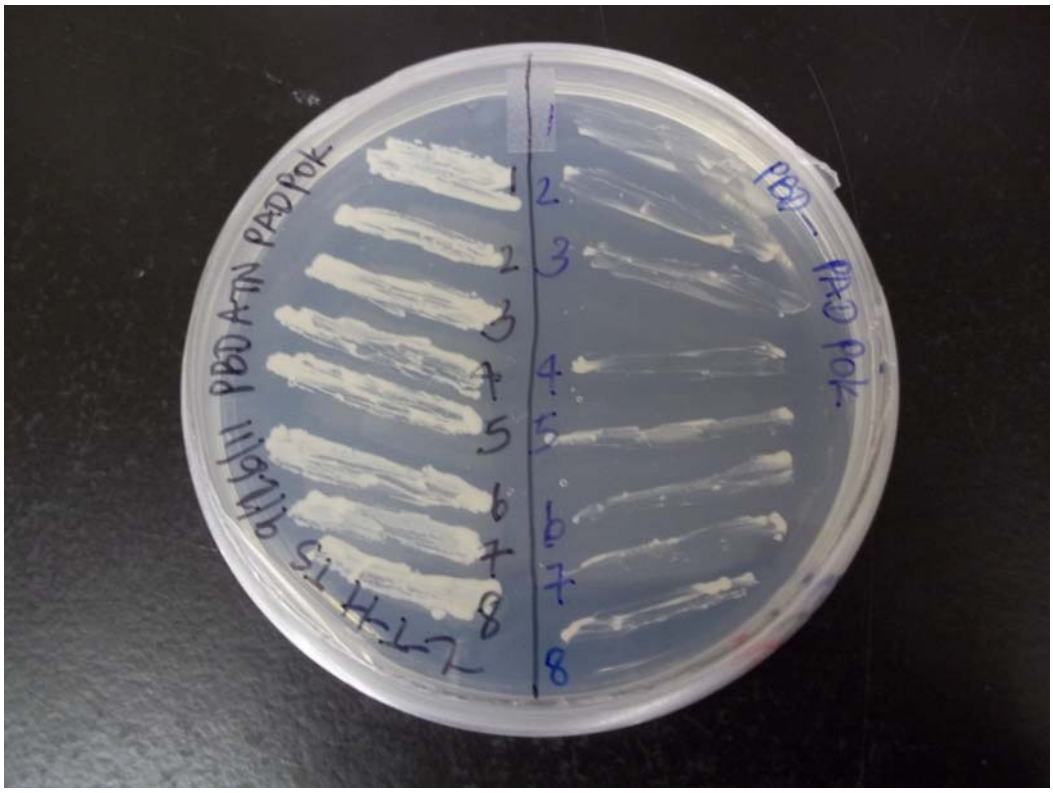


Figure 3. Growth of yeast on CSM-L-T-H for positive and negative controls of the self-activator test. This Figure shows the growth of yeast on media lacking leucine, tryptophan, and histidine in the positive and negative controls for the yeast-two-hybrid self-activator test. This picture was taken four days after the yeast was being streaked onto the CSM-L-T-H media and incubated at 28°C. On the left side of the plate, the eight streaks of yeast contain the pBD with TANGLED cDNA and the pAD with POK1 cDNA. On the right side of the plate, the eight streaks of yeast contain the pBD empty vector with nothing inserted in the multiple cloning site and the pAD with POK1 cDNA.

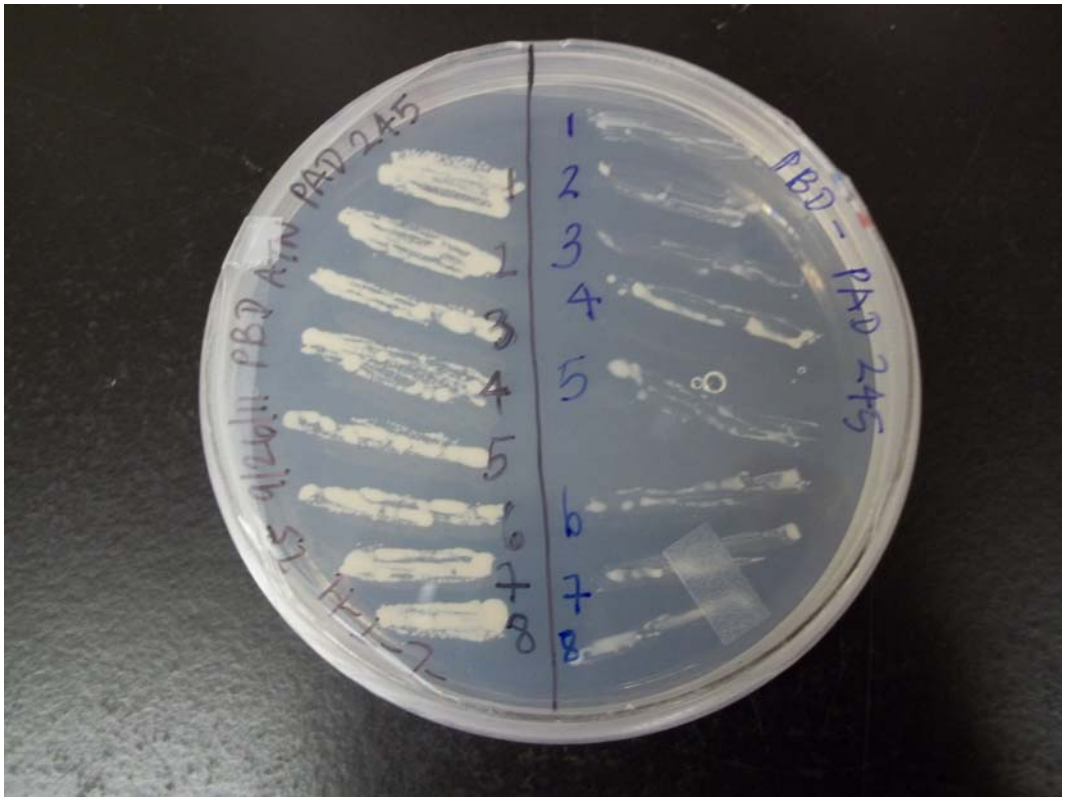


Figure 4. Growth of yeast on CSM-L-T-H as an example of a non-self-activator. This Figure shows the growth of yeast on media lacking leucine, tryptophan, and histidine for a cDNA clone that has been shown to be a non-self-activator during the yeast-two-hybrid self-activator test. This picture was taken four days after the yeast was being streaked onto the CSM-L-T-H media and incubated at 28°C. On the left side of the plate, the eight streaks of yeast contain the pBD with TANGLED cDNA and the pAD with DUF 593 (AT1G70750) cDNA. On the right side of the plate, the eight streaks of yeast contain the pBD empty vector with nothing inserted in the multiple cloning site and the pAD with AT1G70750 cDNA.

Determination of promising candidates for bona fide interaction with TANGLED

For each of the 17 proteins that interacted with TANGLED, I also considered the possibility of it being a false positive generated by yeast-two-hybrid. In other words, I determined whether the protein is a promising candidate for bona fide interaction with TANGLED. I did this by reading about the functions of the protein

and judging if it is logical that this protein contributes to cell division plane orientation. If the functions of a protein are not related with cell division, cytoskeleton, and cell cycle, then it is unlikely that this protein contributes to cell division plane orientation, and I don't consider its interaction with TANGLED in yeast-two-hybrid to be bona fide. I don't consider such a protein to be a promising candidate. If there is no publication on the functions of a protein, I read about the functions of very closely related proteins and made my decisions based on that. An example of a protein that I don't consider to be a promising candidate is PHOTOSYSTEM I SUBUNIT H2; its name implies its function in photosynthesis, which is not directly related with cell division plane orientation, and thus its interaction with TANGLED in yeast-two-hybrid is unlikely to be bona fide. By this process, I determined that of the 17 proteins, nine proteins are promising candidates for bona fide interaction with TANGLED. Table 1 shows which of the proteins are the promising candidates. One of the promising candidates turned out to be the same as the control: POK1. Thus, eight new promising candidates for bona fide interaction with TANGLED are identified (Table 1).

Table 1. Proteins that interact with TANGLED in yeast-two-hybrid assay. Positive transformants were obtained from a yeast-two-hybrid screen of *Arabidopsis thaliana* cDNA library for proteins that interact with TANGLED. The cDNA clones in the pAD plasmids of these positive transformants were tested for self-activation. The identities of cDNA clones that showed no self-activation were determined by sequencing and TAIR BLAST. This table shows the identities of the proteins coded by these cDNA clones. The column "Number of times Appeared" shows the number of times that each protein appeared from the yeast-two-hybrid screen as a non-self-activator cDNA clone. This table also shows whether the protein is considered a promising candidate for bona fide interaction with TANGLED, as determined by its biological function.

Identity of cDNA Clone	TAIR #	Number of times Appeared	Promising candidate for bona fide interaction with TANGLED?
RAB GTPase-activating protein (RAB GAP)	AT3G55020	2	Yes
PHOX2	AT1G62390	5	Yes
Protein of unknown function with domain of unknown function 593 (DUF593)	AT1G70750	3	Yes
Protein of unknown function with domain of unknown function 593 (DUF593)	AT5G16720	2	Yes
KINECTIN-related protein	AT2G17990	1	Yes
OBERON1	AT3G07780	2	Yes
OBERON2	AT5G48160	2	Yes
MYOSIN heavy chain-related protein	AT5G07890	2	Yes
PHRAGMOPLAST ORIENTING KINESIN 1 (POK1)	AT3G17360	1	Yes
MADS-box family protein	AT3G12510	2	No
RUBISCO SMALL SUBUNIT 3B	AT5G38410	2	No
PHOTOSYSTEM I SUBUNIT H2	AT1G52230	1	No
14-3-3 PROTEIN G-BOX FACTOR14 LAMBDA (AFT1)	AT5G10450	1	No
ATU2AF65A	AT4G36690	1	No
WAX2	AT5G57800	1	No
PECTIN LYASE-like superfamily protein	AT3G07820	1	No
ACT DOMAIN REPEATS 9 (ACR9)	AT2G39570	1	No

Testing promising candidates for interaction with different regions of TANGLED

Of the eight new promising candidates, I tested seven of them to see what region of TANGLED each protein interacts with. Rasmussen et al 2011a divided TANGLED into five different regions. The cDNA coding for each region of TANGLED and the cDNA coding for TANGLED with each of these regions deleted were cloned into the pAS vector, which is similar to pBD in that the cDNA is fused with the DNA binding domain of yeast GAL4 (Rasmussen et al., 2011a). The construct pAS 1-132 codes for Region I (amino acids # 1-132) of TANGLED. The construct pAS 126-end codes for amino acids #126-473, which comprises Regions II-V (TANGLED without Region I). The construct pAS 126-229 codes for Region II (amino acids #126-229). The construct pAS no 132-222 codes for amino acids #1-132 and #222-473, which comprises Regions I along with III-V, in other words, TANGLED without Region II. The construct pAS 1-229 codes for Regions I and II (amino acids # 1-229). (Rasmussen et al., 2011a)

For the seven promising candidates, I used yeast-two-hybrid to test whether they interact with Region I (amino acids # 1-132) of TANGLED and/or Region II (amino acids # 126-229), and also whether Region I and/or Region II interaction is necessary and sufficient for the protein to interact with TANGLED. Although I didn't specifically test for the proteins' interaction with Regions III-V (amino acids # 222-473) of TANGLED, it may be inferred from my results whether these proteins interact with Regions III-V of TANGLED because the construct with Region I deleted (the pAS 126-end construct) and the construct with Region II deleted (the pAS 132-222 construct) also code for Regions III-V.

The pAD vectors with the promising candidates' cDNAs were tested for their abilities to interact with the five pAS deletion constructs of TANGLED. These deletion constructs of TANGLED in pAS are not self-activators, as shown in Supplemental Figure S1 of Rasmussen et al 2011a. For the positive control in these tests (for determining if the yeast grew), I used yeast containing pAD with cDNA of the protein being tested and TANGLED cDNA in pBD (pBD TAN, coding for a full length TANGLED); for the negative control (for determining if yeast failed to grow), I used yeast containing empty pBD and pAD with the cDNA of the protein being tested.

The results of these seven proteins' interaction with the deletion constructs of TANGLED are shown in Table 2 and Table 3. Table 2 shows whether yeast with the cDNA clone of each TANGLED interactor in pAD and each TANGLED deletion construct in the pAS grew on media lacking tryptophan, leucine, and histidine. Table 2 also shows the growth of yeast for the positive and negative controls for each TANGLED interactor. Based on the growth or lack of growth of yeast shown in Table 2, I deduced which region(s) of TANGLED are necessary and sufficient for interaction with each of the seven proteins with TANGLED in Table 3.

Table 2. Growth of yeast containing TANGLED interactors as the activating domain and deletion constructs of TANGLED as the DNA binding domain on CSM-L-T-H. Yeast-two-hybrid was used to determine whether seven of the eight new promising candidates interact with Region I and/or II of TANGLED. Each grid in this Table represents yeast that has been transformed with two plasmids: a certain promising candidate's cDNA clone in pAD vector and a certain TANGLED deletion construct in pAS vector or a pBD vector representing the positive or negative control. The name of the protein coded by the cDNA clone in the pAD is shown in each row. The pAS deletion construct of TANGLED or the pBD construct is show in each column. A + sign represents that the yeast containing the two particular plasmids grew on media lacking tryptophan, leucine, and histidine (CSM-L-T-H). A - sign represents that the yeast containing the two particular plasmids failed to grow on CSM-L-T-H.

cDNA clone in pAD	pBD TAN	pBD empty	pAS 1-132 (I)	pAS 126-end (II-V)	pAS 126-229 (II)	pAS no 132-222 (I and III-V)	pAS 1-229 (I+II)
RAB GAP	+	-	+	-	-	+	+
OBERON1	+	-	-	-	-	+	+
OBERON2	+	-	-	-	-	+	+
KINECTIN-related protein	+	-	+	-	-	+	+
DUF593, AT5G16720	+	-	+	-	-	+	+
DUF593, AT1G70750	+	-	-	-	-	+	-
PHOX2	+	-	-	-	-	+	+

Table 3 shows that Region I of TANGLED is necessary and sufficient for the interaction of RAB GAP, the KINECTIN-related protein, and one of the DUF593 proteins (AT5G16720) with TANGLED. For each of these three proteins, the growth of yeast on the positive control and the lack of growth of yeast on the negative control

confirmed that these three proteins are not self-activators (Table 2). The binding of the proteins to Region I and its lack of binding to Regions II-V (pAS 126-end) suggests that Region I is necessary for these proteins to interact with TANGLED (Table 2). The results with the Region II deletion constructs (pAS 126-229 and pAS no 132-222) show that Region I is sufficient for the three proteins' interaction with TANGLED. Thus, Region I of TANGLED is necessary and sufficient for the interaction of RAB GAP, the KINECTIN-related protein, and one of the DUF593 proteins (AT5G16720) with TANGLED.

Table 3 shows that discrepancy exists between the results for OBERON1, OBERON2, and PHOX2. In Table 2, the results for pAS 126-229 (Region II) and pAS 126-end (Regions II-V) show that there is no interaction between the three proteins and Regions II-V (pAS 126-end). The result of the proteins' interaction with the Region II deletion construct (pAS no 132-222) shows that the three proteins bind to Region I of TANGLED. Furthermore, that proteins' interaction with pAS 1-229 (Regions I and II) shows that three proteins binds to Region I. This, however, is in conflict with the data for pAS 1-132. Thus, I conclude that the results for the three proteins' interaction with Region I are inconsistent.

Table 3 also shows inconsistency between the results for the other DUF593 protein (AT1G70750). As Table 2 shows, the results with pAS 1-132 (Region I), pAS 126-229 (Region II), and pAS 1-229 (Regions I and II), show that that there is no interaction between AT1G70750 and Regions I and II. The results for pAS no 132-222 (no Region II) shows that there is interaction between Regions III-V, but the lack of interaction with pAS 126-end (Regions II-V) contradicts this. Thus, I conclude that

there is inconsistent data regarding AT1G70750's interaction with Regions III-V of TANGLED.

Table 3. The seven proteins' interaction with specific Region(s) of TANGLED. Yeast-two-hybrid was used to determine which specific Regions of TANGLED do the seven promising candidates that interact with TANGLED bind to. Regions I is amino acids #1-132 of TANGLED. Region II is amino acids # 126-229. Regions III-V is amino acids # 222-473. The results are deduced from the growth of yeast shown in Table 2. Name of the protein is shown in the first column. The Region(s) of TANGLED that the protein interacts with is shown in the second column. The compartmentalized bars are representative of the five Regions of TANGLED. If a compartment is green, that means that part of TANGLED is necessary and sufficient for the protein to interact with TANGLED. If a compartment is white, that means that there is no interaction between the protein and that Region of TANGLED. If a compartment is pink, there is possible interaction between the protein and that Region of TANGLED, but the results were inconsistent.

Protein	Interacts with which Region of TANGLED?
RAB GAP	
OBERON1	
OBERON2	
KINECTIN-related protein	
DUF593, AT5G16720	
DUF593, AT1G70750	
PHOX2	

Localizations of RAB GAP and PHOX2

These experiments were done in collaboration with Carolyn Rasmussen, a postdoc in the Smith lab. To investigate the possible functions of the two promising candidates RAB GAP and PHOX2 in cell division, we made constructs in which the two proteins were fused to yellow fluorescent protein (YFP). The constructs express the YFP fusion proteins from the 35S promoter (Walker et al., 2007; Earley et al., 2006). The constructs were transformed into *Arabidopsis thaliana* expressing cerulean fluorescent protein (CFP) tagged alpha-tubulin. The YFP and CFP fusion proteins were visualized in dividing cells of seedling root tips. The localized RAB GAP protein is partial length—amino acids #550-778, and the localized PHOX2 is a full length protein. During telophase of a dividing root tip cell of a six-day-old seedling, RAB GAP :: YFP localizes to the maturing cell plate, which is in the space in between the two clusters of the expanding phragmoplast (Figure 5). Similarly, YFP :: PHOX2 also localizes to the cell plate (Figure 6). However, YFP :: PHOX2 is not found at the cell plate until late telophase, when the phragmoplast has almost completely expanded to the cortex of the cell (when t = 10 minutes in Figure 6).

Carolyn Rasmussen is a co-author of Figure 5 (Localization of RAB GAP :: YFP during telophase) and Figure 6 (Localization of YFP :: PHOX2 during telophase) and has given me the permission to use these two Figures in this Thesis. These two Figures have not been published, have not been submitted for publication, and are not currently being prepared for publication.

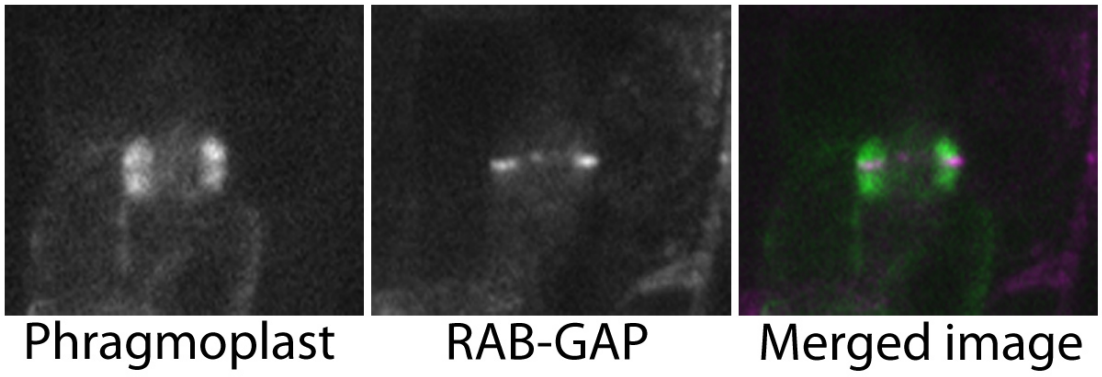


Figure 5. Localization of RAB GAP :: YFP during telophase. CFP :: alpha-tubulin and RAB GAP :: YFP were visualized in a dividing root tip cell in a six-day-old *Arabidopsis thaliana* seedling. The RAB GAP here is a partial length protein, amino acids #550-778. In the merged image, green shows the CFP :: alpha-tubulin and magenta shows the RAB GAP :: YFP. The CFP :: alpha-tubulin took the shape of an expanding late phragmoplast. RAB GAP :: YFP localizes to the cell plate—the area between the two clusters of the phragmoplast. This experiment was done in collaboration with Carolyn Rasmussen, a postdoc from the Smith lab. Carolyn Rasmussen is also a co-author of this Figure.

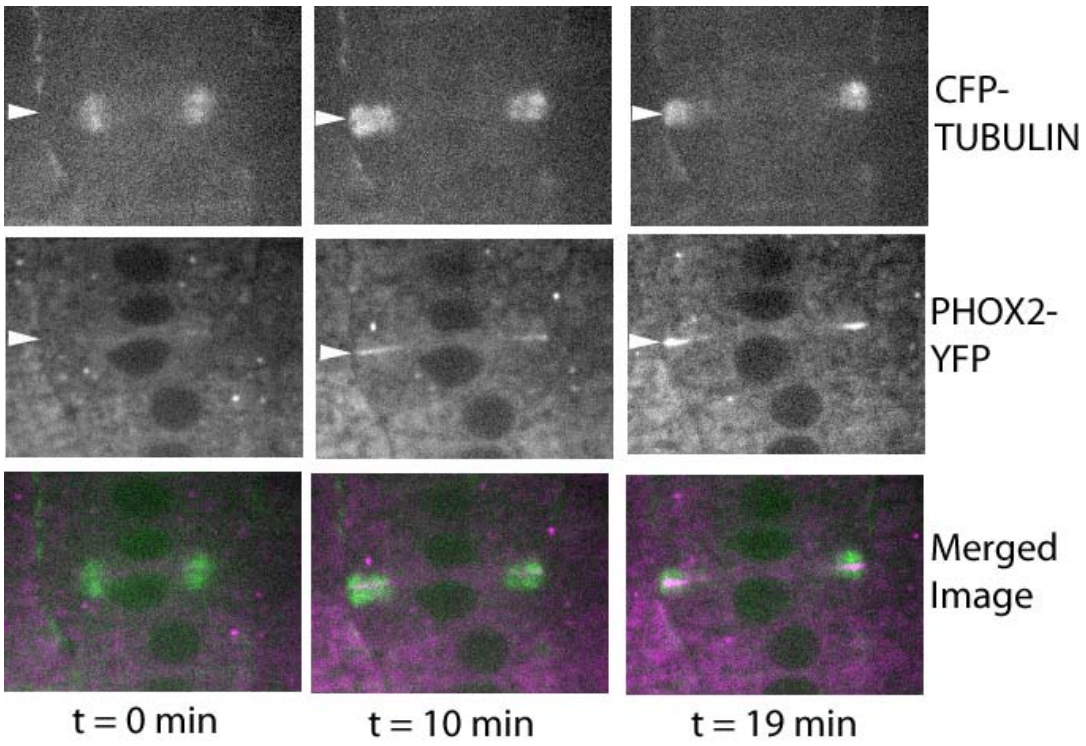


Figure 6. Localization of YFP :: PHOX2 during telophase. CFP :: alpha-tubulin and YFP :: PHOX2 were visualized in a dividing root tip cell in an *Arabidopsis thaliana* seedling. The PHOX2 here is a full length protein. In the merged images, green shows the CFP :: alpha-tubulin and magenta shows YFP :: PHOX2. The CFP :: alpha-tubulin took the shape of a late expanding phragmoplast. After t = 10 minutes, YFP :: PHOX2 localizes to the cell plate—the area between the two clusters of the phragmoplast (arrows). This experiment was done in collaboration with Carolyn Rasmussen, a postdoc from the Smith lab. Carolyn Rasmussen is also a co-author of this Figure.

DISCUSSION

Brief summary of results and their potential impact on understanding TANGLED function

Using yeast-two-hybrid assay, I found eight new proteins that are promising candidates for bona fide interaction with TANGLED. They are RAB GAP, PHOX2, two different proteins of unknown function with the same domain of unknown function 593 (DUF593), KINECTIN-related protein, OBERON1, OBERON2, and MYOSIN heavy chain-related protein (Table 1). Also using yeast-two-hybrid, I found that the first 132 amino acids of TANGLED are necessary and sufficient for its interaction with RAB GAP, KINECTIN-related protein, and one of the DUF593 proteins (AT5G16720), (Tables 2 and 3). For RAB GAP and PHOX2, localization experiments involving fusion with yellow fluorescent protein revealed that they localize to the cell plate in dividing *Arabidopsis thaliana* cells (Figures 5 and 6). As will be discussed, my results along with results from published literature suggest that TANGLED is a possible regulator of some of these proteins, some of these proteins may also be involved in TANGLED localization, and some of them may function in the same pathway as TANGLED.

Possible causes of inconsistent results

For OBERON1, OBERON2, and PHOX2, my results regarding the proteins' interaction with Region I of TANGLED were contradictory (Tables 2 and 3). All three of these proteins interacted with TANGLED Regions I+III-V and TANGLED Regions

I+II. They also showed no interaction with Regions II-V. Altogether, these imply that they interact with Region I. However, none of them interacted with TANGLED Region I alone. For one DUF593 protein (AT1G70750), my results regarding the protein's interaction with TANGLED Regions are similarly contradictory. This protein interacted with TANGLED regions I+III-V and showed no interaction with Region I and Region II. Altogether, these imply that this protein interacts with Regions III-V; however, it failed to interact with TANGLED Regions II-V, which contains Regions III-V (Tables 2 and 3).

Some sources of error contributing to these contradictory results may include the variability in the amount of inoculum of yeast and also the variability in the depth of media used. I should repeat this experiment, making sure that I used the similar amount of inoculum of yeast and that my media is of a similar depth throughout the experiment. However, it is also likely that the particular conditions of the yeast cell failed to allow the interaction of those particular deletion constructs of TANGLED (pAS 1-132, pAS 126-end) with the proteins. Perhaps the particular conditions within the yeast are not allowing the TANGLED fragments to fold properly to bind to a particular region on those proteins. In this case, a method other than yeast-two-hybrid should be used to detect the interaction between the proteins and these Regions of TANGLED. Some of these methods include fluorescence resonance energy transfer (FRET) and co-immunoprecipitation. For the DUF593 protein AT1G70750, I should also repeat the experiment with a new deletion construct coding for amino acids #222-473 (Regions III-V) of TANGLED to test directly for the protein's interaction with Regions III-V of TANGLED.

TANGLED is a possible regulator of RAB GAP

My results show that RAB GAP is an interactor of TANGLED (Table 1) and that Region I of TANGLED is also necessary and sufficient for its interaction with RAB GAP (Tables 2 and 3). Figure 5 shows that amino acids #550-778 of RAB GAP localizes to the nascent cell plate during telophase of a dividing root tip cell of *Arabidopsis*. Since TANGLED is localized as a ring at the cortical division site (Walker et al., 2007), RAB GAP and TANGLED could co-localize and interact once the phragmoplast, along with the cell plate, expands to the cortex. Based on the localization of RAB GAP, I believe that RAB GAP is a promising candidate for bona fide interaction with TANGLED.

Currently, very little is known about the function of RAB GAP. RAB GAP inactivates RAB GTPases by exchanging GTP for GDP (Nielsen et al., 2008). RAB GTPases are involved in vesicle trafficking (Nielsen et al., 2008). Chow et al 2008 showed that RAB-A2 and RAB-A3 (two subclasses of RAB GTPase) localize to the cell plate, especially to the expanding edges of the cell plate as the phragmoplast and cell plate expand toward the cortex of the cell, in dividing root tip cells of *Arabidopsis* seedlings. Thus, RAB GTPases localize similarly to the RAB GAP fragment we localized. In *Arabidopsis*, RAB-A2 dominant-inhibitory mutants that bind only GDP or bind no nucleotides at all are always in an inactive state and they show multinucleated epidermal root cells, indicating that the cells fail to undergo cytokinesis (Chow et al., 2008). The dominant gain-of-function RAB-A2 *Arabidopsis* mutants only bind to GTP and are always active and they show no multinucleated cells (Chow et al., 2008). The dominant-inhibitory RAB-A2 mutants also show severely

abnormal cell expansion, highly irregular cell files, and incomplete cell walls (Chow et al., 2008). This mutant phenotype is similar to that of the *tangled* maize mutants, but in the *tangled* mutant the cell wall is complete (Cleary and Smith, 1998).

Based on my results and the results of Chow et al 2008, I hypothesize that TANGLED inactivates RAB GAP, and that the inactivation of RAB GAP by TANGLED at the conclusion of cytokinesis attaches the cell plate to the cortical division site. The dominant-inhibitory RAB-A2 mutants have incomplete cell walls (Chow et al., 2008). This shows that RAB-A2, in its active form, is necessary for the complete formation of the cell wall. This is logical because RAB GTPases are involved with vesicle trafficking and that the formation of the cell plate requires the fusion of Golgi-derived vesicles (Nielsen et al., 2008; Samuels et al., 1995). RAB GAP inactivates RAB-A2 (Nielsen et al., 2008). Thus, inactivation of RAB GAP would contribute to the complete formation of the cell wall. The complete formation of the cell wall involves the attachment of the cell plate to the cortical division site at the conclusion of cytokinesis. Since RAB GAP and TANGLED co-localize and interact with each other at the conclusion of cytokinesis when the cell plate attaches to the cortical division site, it is possible that at the conclusion of cytokinesis, TANGLED inactivates RAB GAP so that there would be more active RAB-A2 to attach the cell plate to the cortical division site, contributing to the complete formation of the cell wall. Since RAB GAP interacts only with Region I of TANGLED, it is possible that Region I of TANGLED inactivates RAB GAP. Many experiments will be needed to test this hypothesis.

HSP90 and PHOX2 form a complex of which TANGLED is a possible client

My results show that PHOX2 and TANGLED interact (Table 1). There is the possibility that PHOX2 interacts with Region I of TANGLED, though the results on this were contradictory, as discussed earlier (Tables 2 and 3). I believe that PHOX2 is a promising candidate for interaction with TANGLED because PHOX2 localizes to the cell plate during late telophase, when phragmoplasts have almost expanded all the way to the cortex of the cell (Figure 6). As phragmoplast expands to the cortex of the cell, PHOX2 should briefly contact TANGLED since TANGLED is localized as a cortical ring at the division site (Walker et al., 2007).

PHOX2 belongs to a family of proteins that contain the tetratricopeptide repeat domain that interacts with the EEVD motif at the C-terminus of HSP90 (Heat shock protein 90), (Prasad et al., 2010). Prasad et al 2010 identified PHOX2 as a co-chaperone of HSP90. HSP90 is a highly conserved protein in eukaryotic cells (Krishna and Gloor, 2001). HSP90 form complexes with its co-chaperones, and these complexes regulate the folding, localization, activation, and protein-protein interactions of wide range of proteins including proteins that are involved in signal transduction and cell cycle regulation (Prasad et al., 2010; Krishna and Gloor, 2001; Pearl et al., 2008). The proteins that HSP90 regulates are called its client proteins. In *Arabidopsis thaliana,* HSP90-7 (AT4G24190) is predicted to localize to the endoplasmic reticulum; HSP90-7 has a signal sequence on its N-terminus as well as a KDEL endoplasmic reticulum retention motif on its C-terminus (Krishna and Gloor, 2001). Moreover, HSP90-7 is very similar to other HSP90 plant orthologs that are specific to the endoplasmic reticulum (Krishna and Gloor, 2001).

Based all these results and also my result identifying PHOX2 as an interactor of TANGLED in the yeast-two-hybrid system (Table 1), I hypothesize that PHOX2 along with HSP90-7 creates a complex of which TANGLED is a possible client. Because HSP90-7 localizes to the endoplasmic reticulum, there is the possibility that it can be found in the cell plate since large amounts of endoplasmic reticulum can be found in the phragmoplast as well as the cell plate during telophase (Gupton et al., 2006). Since PHOX2 localizes to the cell plate during late telophase and because it is a co-chaperone of HSP90, it is likely that both PHOX2 and HSP90-7 localize to the cell plate during late telophase and form a complex there. Since PHOX2 also interacts with TANGLED, it is possible that the binding of PHOX2 to TANGLED brings TANGLED to the complex formed by HSP90 and PHOX2. Because one of HSP90's diverse functions is mediating the localization of proteins, it is possible that HSP90 changes the conformation of TANGLED so that TANGLED disappears from the division site upon the completion of cytokinesis.

My results for PHOX2's interaction with Region I of TANGLED are inconsistent (Tables 2 and 3). However, if PHOX2 indeed interacts with Region I of TANGLED, this would support my hypothesis that HSP90 changes the conformation of TANGLED so that TANGLED disappears from the cortical division site upon the completion of cytokinesis. This is because Region I of TANGLED is needed for TANGLED localization to division site from anaphase to cytokinesis (Rasmussen et al., 2011a). If PHOX2 interacts with Region I, then it is likely that PHOX2 brings Region I of TANGLED into HSP90 so that HSP90 can change the Region I of TANGLED in a way that disallows TANGLED's localization to the cortical division

site, thus making TANGLED disappear from the cortical division site as cytokinesis is completed. However, before I can make this assumption, I have to confirm that PHOX2 indeed interacts with Region I.

Many additional experiments are needed to test my hypothesis that TANGLED is a client of a PHOX2/HSP90 complex. It will be necessary to observe the localization of HSP90-7 in dividing *Arabidopsis* cells to see if it co-localizes with PHOX2 and TANGLED during late telophase. It will also be necessary to test for the interaction between TANGLED and HSP90, since a direct interaction between TANGLED and HSP90 would be required for HSP90 to change the conformation of TANGLED. This can be done by yeast-two-hybrid or co-immunoprecipitation. In *Arabidopsis* root, shoot, and leaf tissues, heat stress induces the expression of PHOX2 (Prasad et al., 2010). Thus, it will be important to check the mRNA levels of PHOX2 in dividing *Arabidopsis* cells to see if cell division activates the expression of PHOX2 as well.

AT1G70750 and AT5G16720 are two proteins of unknown function with the same domain of unknown function 593 (DUF593)

My results have also shown that two proteins of unknown function with the same domain of unknown function 593 (DUF593) — AT1G70750 and AT5G16720— interact with TANGLED (Table 1). Proteins with domain of unknown function 593 (DUF593) are plant-specific proteins. The DUF593 region is highly conserved among plants (Holding et al., 2007). In proteins with the DUF593 region, the DUF593 is either on the C-terminus of protein or N-terminus of the protein (Holding et al., 2007).

Currently, there is no publication on the function of DUF593 in *Arabidopsis*. However, in maize, the gene *FLOURY1* codes for a transmembrane protein with the DUF593 domain at the C-terminus of the protein; this protein (FL1) localizes to the endoplasmic reticulum that surrounds the zein protein body (zein protein body is important for kernel maturity in maize), and it is necessary for formation of the vitreous endosperm (Holding et al., 2007).

The full length DUF593 protein coded by the *FLOURY1* gene is 55% homologous to the DUF593 proteins in *Arabidopsis*, and the only region of homology between the FL1 protein and the *Arabidopsis* DUF593 proteins is the DUF593 region (Holding et al., 2007). Using CLUSTALW I aligned the full length protein sequences of AT1G70750 and AT5G16720 together and found that 33% of the two proteins' amino acids are identical. Also using CLUASTALW, I aligned the DUF593 regions of the two proteins together and found that 66% of the amino acids in the DUF593 regions of the proteins are identical.

According to the phylogenic tree in Figure 3 of Holding et al 2007, both AT5G16720 and AT1G70750 have the DUF593 region near the C-terminus and a signal sequence near the N-terminus. After doing a search on the proteins' topology on Aramemnon (http://aramemnon.uni-koeln.de/), I found that each protein has at least one transmembrane domain on the N-terminus. In addition, I didn't find any KDEL or HDEL endoplasmic reticulum retention sequence in the protein sequences of either proteins. These sequence features predict that the two DUF593 proteins are secretory proteins that initially localize to the endoplasmic reticulum as they are being synthesized and can be transported from there to the Golgi (Jurgens, 2004). In dividing

cells, the DUF593 proteins are most likely transported to the cell plate because the default pathway for the trafficking of secretory proteins in dividing cells is from the Golgi to the cell plate (Jurgens, 2004). Just like RAB GAP and PHOX2, the two DUF593 proteins may localize to the cell plate throughout mitosis and cytokinesis, and then co-localize and interact with TANGLED at the end of cytokinesis when the phragmoplast expands to the cortex. Localization of both proteins is needed to test this hypothesis.

I found that Region I of TANGLED is necessary and sufficient for AT5G16720's binding to TANGLED. According to Rasmussen et al 2011a, Region I of TANGLED is needed for TANGLED to localize to the division site from anaphase to cytokinesis. Thus, it is possible that AT5G16720 plays a role in mediating TANGLED localization during these phases. If AT5G16720 localizes to the cell plate, then it may only affect the disappearance of TANGLED from the cortical division site at the end of cytokinesis since the cell plate doesn't contact TANGLED until end of cytokinesis.

KINECTIN-related protein (AT2G17990) may bind POK1 to contribute to the localization of TANGLED

KINECTIN-related protein (AT2G17990) is another protein that interacts with TANGLED in yeast-two-hybrid (Table 1). Currently, there is no publication on the function of the KINECTIN-related protein or on the function of KINECTIN in plants. In mammals, there is also a protein called kinectin. Human kinectin is 1300 amino acids long while the *A. thaliana* KINECTIN-related protein is only 338 amino acids

long. Using CLUSTALW, I aligned the protein sequences of the human kinectin (GenBank: AAB65853.1) and the KINECTIN-related protein and found that 70 of the 338 amino acids (20%) in the KINECTIN-related protein are identical to the amino acids in the human kinectin.

In human cells, kinectin binds directly to kinesin and attaches membrane bound organelles to kinesin to allow the motility of these organelles (Ong et al., 2000). Also in human cells, kinectin is a resident transmembrane protein on the endoplasmic reticulum and its interaction with kinesin allows the endoplasmic reticulum to move along microtubules to promote the formation of the focal adhesion on the cellular lamellae; this process is necessary for the motility of the cell, and in the case of cancer cells, necessary for metastasis (Zhang et al., 2010).

After doing a search on the protein topology on Aramemnon (http://aramemnon.uni-koeln.de/), I found that the KINECTIN-related protein has no transmembrane domains. Furthermore, I found no KDEL or HDEL endoplasmic reticulum retention signal in the protein's sequence. This suggests that unlike the human kinectin, this KINECTIN-related protein is not an endoplasmic reticulum membrane resident protein. Using Signal-3L, (http://www.csbio.sjtu.edu.cn/bioinf/Signal-3L/), I also found no signal sequence in this protein's sequence. This indicates that this protein is not localized in the endoplasmic reticulum, and it is therefore not a secretory protein. Although this protein differs from the human kinectin in this aspect, it is still unknown whether this protein binds KINESIN.

If this KINECTIN-related protein is KINESIN binding, there is the possibility that it binds to POK1, a kinesin that interacts with TANGLED and is necessary for TANGLED localization from anaphase to cytokinesis (Muller et al., 2006; Walker et al., 2007). Using yeast-two-hybrid to determine if POK1 interacts with the KINECTIN-related protein may be one way to test this.

My result shows that Region I of TANGLED is necessary and sufficient for this KINECTIN-related protein to interact with TANGLED (Tables 2 and 3). Both POK1 and Region I of TANGLED are required for TANGLED localization to the division site from anaphase to cytokinesis (Rasmussen et al., 2011a; Walker et al., 2007). Thus, if the KINECTIN-related protein binds to POK1 and Region I of TANGLED, then it may play a role in mediating the localization of TANGLED to the division site from anaphase to cytokinesis.

OBERON1 and OBERON2 may function in the same pathway as TANGLED

My results show that two very similar proteins, OBERON1 and OBERON2, are interactors of TANGLED (Table 1). OBERON1 has also been identified as an interactor of TANGLED by a large scale interactomics study involving yeast-two-hybrid (Mukhtar et al., 2011). OBERON1 and OBERON2 protein sequences are 75% identical with each other (Saiga et al., 2008). OBERON1 and OBERON2 allow *Arabidopsis* to maintain meristems by maintaining stem cell population (Saiga et al., 2008). OBERON1 and OBERON2 also control the transcription of the auxin genes to regulate growth and development (Thomas et al., 2009).

OBERON1 and OBERON2 are two proteins that are expressed in young *Arabidopsis* tissue such as heart-stage embryos and roots of seedlings (Saiga et al., 2008; Thomas et al., 2009). The two proteins' expression in young tissue with many actively dividing cells makes them promising candidates for bona fide interaction with TANGLED.

The *Arabidopsis obe1obe2* double mutants also have chaotic cell divisions of the hypophysis with disorganized tissue as the result (Thomas et al., 2009; Saiga et al., 2008). The phenotype of disorganized cell divisions is the same as that in *Arabidopsis tangled* mutants, though the *obe1obe2* phenotype is more severe (Walker et al., 2007). This may mean that OBERON1 and OBERON2 along with TANGLED are involved in the same pathway such that if OBERON1 and OBERON2 malfunctions, TANGLED malfunctions as well. Additional experiments are needed to test if OBERON and TANGLED are in the same pathway and if OBERON1 and OBERON2 act upstream or downstream of TANGLED. Because of this published mutant phenotype of *obe1obe2*, I believe that OBERON1 and OBERON2 are promising candidates for bona fide interaction with TANGLED.

Arabidopsis double mutants of *obe1obe2* have smaller cotyledons and roots than wild type (Saiga et al., 2008; Thomas et al., 2009). This phenotype is similar to the phenotype of the *pok1pok2* double mutants (Muller et al., 2006). Furthermore, the chaotic cell division phenotype of the *obe1obe2* mutants is also similar to *pok1pok2* double mutants (Muller et al., 2006). POK1 and POK2 are two kinesins that are necessary for TANGLED to localize to the division site from anaphase to cytokinesis, and POK1 also interacts with TANGLED (Walker et al., 2007; Muller et al., 2006).

This shows that the OBERON and POK possibly have similar functions. Perhaps TANGLED is involved the functions of OBERON1 and OBERON2 as well. POK1 and Region I of TANGLED both are involved in helping TANGLED localize to the division site from anaphase to cytokinesis (Walker et al, 2007; Rasmussen et al., 2011a). My results for OBERON1 and OBERON2's interaction with Region I is inconsistent; but if OBERON1 and OBERON2 indeed interact with Region I, then it is possible that OBERON1 and OBERON2 are involved with helping TANGLED localize to the division site from anaphase to cytokinesis. However, I need to confirm the interaction of OBERON1 and OBERON2 with Region I before I can make this assumption.

TANGLED as an inhibitor of MYOSIN heavy chain-related protein: overexpression of MYOSIN heavy chain-related protein may cause abnormal cell shape in plants

MYOSIN heavy chain-related protein (AT5G07890) was also identified to be a TANGLED interactor (Table 1). There are many types of myosins in plants. They're required for organelle movement, endoplasmic reticulum tubule growth, and F-actin arrangement (Sparkes, 2011).

I believe that this protein is a promising candidate for bona fide interaction with TANGLED because this protein, when overexpressed, causes abnormal cell shape in fission yeast (Xia et al., 1996). The Xia et al 1996 paper identified *Arabidopsis* cell-cycle related, cell polarity related, and cytoskeletal proteins using fission yeast: they transformed yeast with an *A. thaliana* cDNA library that will

overexpress proteins, observed for yeast with abnormal cell shape, and then identified the cDNA clone that the abnormal yeast carried; a myosin heavy chain-like protein was one of the proteins identified (Xia et al., 1996). Since the overexpression of this protein causes abnormal cell shape in fission yeast, it is possible that its overexpression causes abnormal cell shape in *Arabidopsis* by acting on a homologous pathway. If the overpression of this protein leads to abnormal cell shape in *Arabidopsis*, then TANGLED may be an inhibitor of this protein or that this protein may be an inhibitor of TANGLED.

Additional experiments are needed to give a better understanding on how this protein is involved in the functions of TANGLED. One future experiment is to use yeast-two-hybrid to see which region of TANGLED this protein binds to. Also, it would be interesting to measure the expression level of TANGLED in the mutants to see if the overpression of this protein has any impact on TANGLED expression level.

Future directions

One future experiment is to localize the promising candidates that have not been localized so far. This includes the two DUF593 proteins (AT1G70750 and AT5G16720), the KINECTIN-related protein, and MYOSIN heavy chain-related protein. Localization of these proteins in dividing cells will reveal the phase of mitosis and cytokinesis in which these proteins interact with TANGLED, giving a better understanding of how they are involved in the localization and function of TANGLED.

Though there is already localization data for RAB GAP, OBERON1, and OBERON2, it is nonetheless important to localize them again to address specific

questions about their localization during mitosis and cytokinesis. For RAB GAP, a full length protein should be localized since the localization of the partial length protein may differ from that of the full length protein. In young root tips, the localization of OBERON1 and OBERON2 is in the nucleus of the cells (Thomas et al., 2009). However, there may or may not be any dividing cells in the root tips that were observed. Thus, it is still necessary to localize OBERON1 and OBERON2 to assess their localization during mitosis and cytokinesis.

I have already cloned a genomic region (from the start to stop codon, without stop codon, with introns) of the DUF593 protein AT1G70750 into pEZRK-LNY, which will tag the C-terminus of the protein with yellow fluorescent protein (Walker et al., 2007). I have transformed this construct into *Arabidopsis thaliana* plants that already have the alpha-tubulin tagged with cerulean fluorescent protein. The cerulean fluorescent protein tagged tubulin within those plants will allow me to match the localization of the AT1G70750 to a particular stage of mitosis and cytokinesis and judge if AT1G70750 localizes to the division site. I have also transformed a YFP :: KINECTIN-related protein construct into *Arabidopsis thaliana* with CFP :: alpha-tubulin. Once the transformants are ready, I will observe the localization of these two proteins.

Another future experiment is to observe the mutant phenotypes of the promising candidates of which there have been no recorded mutant phenotypes, which include the RAB GAP, PHOX2, two DUF593 proteins, the KINECTIN-related protein, and the MYOSIN heavy chain-related protein. The underexpression/underactive and overexpression/overactive protein mutants for each protein can be observed for

orientation of preprophase bands, spindles, and phragmoplast. If the underexpression/underactive mutants have misoriented cell division planes, then it can inferred that the active form of the protein is necessary for proper cell division plane orientation, thus it is possible that either TANGLED activates that protein or the protein activates TANGLED. If the overexpression/overactive mutants have misoriented cell division planes, then the active form of the protein inhibits proper division plane orientation, so it is possible that either TANGLED inhibits that protein or vice versa. This experiment is particularly useful for testing my hypothesis of that the overpression of MYOSIN heavy chain-related protein causes abnormal cell shape in plants and that TANGLED may be an inhibitor of this protein.

The underexpression mutants of each protein can be generated by RNA interference. I have already transformed a construct containing an amiRNA for knocking out both of the DUF593 proteins into *Arabidopsis thaliana* plants that already have the alpha-tubulin tagged with cerulean fluorescent protein. I will be observing the cytoskeletal structures in the dividing cells of the transformants once they are ready.

Another future experiment is to observe the localization of TANGLED throughout mitosis and cytokinesis in the underexpression and overexpression mutants of each promising candidate, and look for any changes in the localization of TANGLED. This can be done for all eight new promising candidates. This experiment will allow me to identify if any of the proteins is necessary for maintaining TANGLED localization at the cortical division site throughout mitosis and cytokinesis or for promoting the disappearance of TANGLED from the cortical division site at the

end of cytokinesis. This experiment is especially important for testing my hypothesis that PHOX2 and the DUF593 protein AT5G16720 promote the disappearance of TANGLED from the division site at the end of cytokinesis. If TANGLED fails to disappear from the division site after cytokinesis in the mutants, then it is possible that the two proteins function in promoting the disappearance of TANGLED from the division site upon the completion of cytokinesis.

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