主論文

Functional analysis of Haspin kinase role in plant cells

（植物細胞におけるハスピンキナーゼの機能解析）

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ABSTRACT

Progression of the cell division is controlled by phosphorylation of various proteins, which triggers transitions from one stage to another. There are several kinase families known, such as CDKs, Plks, and Aurora kinases, involved into regulating cell division. Collaboration between kinases and other factors forms a highly complex, but remarkably accurate system, that orchestrates the events of cell division in time and space.

Proteins from Haspin family have been found in various eukaryotes; however, Haspin kinase role in regulating cell division was mainly investigated in animal cells. The most well-known function of Haspin kinase is phosphorylation of histone H3 at Thr3, which promotes centromeric Aurora B localization to regulate chromosome segregation. Nevertheless, recent studies strongly suggest that Haspin role in regulating cell division is not restricted to the histone phosphorylation, but can include other substrates and factors, possibly related to cytokinesis (Panigada et al. 2013; Maiolica et al. 2014).

Although AtHaspin was identified as histone H3Thr3 kinase in *Arabidopsis thaliana* (Kurihara et al. 2011; Ashtiyani et al. 2011), little is known about Haspin role in plant regulatory network. Current research purpose was further investigation of Haspin kinase in plant cells to shed new light on the plant regulatory network. I combined live cell imaging and the functional analysis, to learn more about Haspin functions, substrates and their down-stream mechanisms.

In this study, NtHaspin from BY-2 tobacco cells was identified and characterized as histone H3Thr3 and Thr11 kinase. A small molecule 5-Iodotubercidin (5-ITu), which was reported as an ATP-competitive inhibitor of

human Haspin kinase (Wang et al. 2012), was used to investigate NtHaspin kinase function. 5-ITu significantly reduced phosphorylation levels both *in vitro* and *in vivo.* Inhibitor treatment in BY-2 plant cell culture led to decreased mitotic index and prolonged mitosis. It was demonstrated that inhibition of NtHaspin kinase in BY-2 cells delayed chromosome alignment during prometa- and metaphase stages of mitosis. NtHaspin inhibition also prevented the centromeric localization of NtAurora3 kinase (NtAUR3) kinase and disrupted its function. This suggested that NtHaspin kinase plays a role in the specific positioning of NtAUR3 on chromosomes in plant cells, a function conserved in animals. The results also indicated that NtHaspin and NtAUR3 kinases are involved in the same pathway, which regulates chromosome alignment during prometa-/metaphase. Remarkably, NtHaspin inhibition by 5-ITu also led to severe cytokinesis defect, resulting in binuclear cells with a partially formed cell plate. While many components are involved into cell plate formation and expansion, 5-ITu treatment did not affect microtubules, AUR1/2, or the NACK-PQR pathway. However, it did alter the distribution of actin filaments on the cell plate. Although actin filaments are prominent component of the phragmoplast, their function in cytokinesis remains unclear. These results indicate that NtHaspin kinase can be a part of a novel cytokinesis-related pathway, which also includes actin filaments.

To summarize, this study showed that NtHaspin kinase is an important factor in regulating cell division in plant cells. Presumably, NtHaspin kinase has several functions in mitosis: initially, during prometa/metaphase it promotes centromeric localization of NtAUR3 kinase and contributes to chromosome alignment, further NtHaspin is involved into regulating late cell plate expansion during cytokinesis.

INTRODUCTION

Cell division in plant and animal cells

Cell division is a process by which a parent cell divides into two or more daughter cells. In eukaryotes, there are two distinct types of cell division: meiosis, a reductive cell division, whereby the number of chromosomes in the daughter cells is reduced by half to produce haploid gametes, and mitosis, whereby daughter cells each contain genetic material identical to the original cell. Mitosis is essential part of basic life events: growth and development, asexual reproduction, regeneration etc. Cells duplicate and condense their DNA prior to entering mitosis. Commonly, two stages of cell division are distinguished: nuclear division, which includes even distribution of genetic material, and cytokinesis, when parental cell divides the cytoplasm to produce two daughter cells. Nuclear division can be subdivided into six distinct phases: prophase, prometaphase, metaphase, anaphase and telophase.

Nuclear division usually, yet not always, is followed by cytokinesis. It should be noted that in some cells nuclear division and cytokinesis occur separately, forming single cells with multiple nuclei, for instance in some fungi, slime molds, coenocytic algae etc (Lilly and Duronio 2005; Nagl 1978). Cytokinesis process is drastically different between animal and plant cells. Animal cells divide via cleavage furrow, containing a contractile ring. It develops on site of the metaphase plate, separating two daughter nuclei. Vesicle trafficking from Golgi apparatus is essential part of cytokinesis process in both animal and plant cells (Albertson et al. 2005). In plant cells vesicle guided by MT merged into the cell plate in the cell equator. Cell plate

expansion is guided by phragmoplast, and array of MT and actin filaments. Eventually cell plate fuses with peripheral cell borders and matures into the new cell wall, separating two daughter cells. Typical plant cell division is presented at Figure 1. Cytokinesis is final step of cell division, generating two daughter cells with a complete copy of the genome of its parent cell.

Cell division is a complicated and complex process, with strict regulation, of which failure leads to serious diseases and disorders of growth and development (Kastan and Bartek 2004; Normand and King 2010). However, many regulatory elements studied in animals are unknown players in the field of plant mitosis. Moreover, plant cell division, while sharing main stages of eukaryotic mitosis, is distinguished by preprophase band, cytokinesis etc. (Cyr and Fisher 2012). The compound cellular machinery required to build a new cell wall, taken together with importance of timing and position, suggests that plants have evolved unique regulatory network to maintain proper cytokinesis.

It is considered that the central role of mitosis regulation belongs to phosphorylation in mitotic checkpoints, spindle function, chromosome segregation and cytokinesis (Nigg 2001). Signaling within and between the molecules that control these events allows for coordination via checkpoints and ensures that the cell division events occur in the proper order. Several mitotic kinases having these functions are

known to be particularly important: Aurora kinases (Demidov et al. 2005; Kawabe et al. 2005), cyclin-dependent kinases etc (Figure 2).

Current opinion on Haspin kinase functions and interactions during mitosis

Haspin belongs to atypical protein kinase family, conserved in many eukaryotic lineages including animals, fungi and plants (Tanaka et al. 1999; Higgins 2001). Originally, the gene and protein were identified in male germ cells of mice, thereafter named germ cell-specific gene 2 (GSG2) and haploid cell-specific protein kinase (Haspin) (Tanaka et al. 1994; Tanaka et al. 1999). Mammalian Haspin proteins have a serine/threonine kinase activity, however up to date the major well-characterized substrate in animal cells is histone H3 at Thr3. In animal and plant cells, several residues of histone H3 are phosphorylated during mitosis, including T3, S10, T11 and S28 (Dai and Higgins 2005; Houben et al. 2007). These modifications are often distinguished in time and spatially. In animal cells, phosphorylated histone H3 at Thr3 (H3T3ph) is concentrated at inner centromeres during prometaphase. H3T3ph signal weakens during anaphase and cannot be detected in telophase cells (Polioudaki et al. 2004; Markaki et al. 2009).

In animal cells, Haspin phosphorylates histone H3 at Thr3, which acts as a docking site for chromosome passenger complex (CPC) with Aurora B kinase as enzymatic subunit as shown in Figure 3 (Wang et al. 2010; Kelly et al. 2010; Yamagishi et al. 2010). Consequently, Haspin knockdown by RNAi prevents proper chromosome alignment at the metaphase plate (Dai et al. 2005; Higgins 2010). Several studies report that chemical inhibition of Haspin counteracts with centromeric localization of Aurora B leading to impaired chromosome congression and spindle assembly checkpoint (SAC) override (De Antoni et al. 2012; Wang et al. 2012). These findings provide strong support for the hypothesis that phosphorylation of H3 at Thr3 provides spatial information for CPC and Aurora B recruitment.

Recently there is growing evidence that Haspin function is not restricted to phosphorylation of H3 at T3. Study by Maiolica et al. (2014), presents a profound investigation of consequences of Haspin chemical inhibition by 5-ITu in mitotic cells. Around 4000 phosphorylation sites in chromatin-associated proteins were quantified after Haspin inhibition, and MacroH2A Ser137 mitotic phosphorylation was verified as due to Haspin activity. The same study pursued *in vitro* kinase reaction with Haspin kinase domain with degenerated peptide library and reported the preferential recognition motif for potential Haspin substrates as A/V-R-T/S-K(X-noD/E). Based on computational analysis and *in vitro assay* another very likely substrate of Haspin is CENP-T which plays role in kinetochore assembly (Maiolica et al. 2014).

Activation of Haspin kinase early in mitosis is triggered by Cdk1-Polo1 kinase pathway. CycB-Cdk1 phosphorylates Haspin and promotes Haspin binding to Polo1 kinase (Zhou et al. 2014). After initial trigger CPC-Aurora B phosphorylation via feedback loop amplifies the signal as shown in Figure 4 (Wang et al. 2011; Qian et al. 2013). Kinetochore kinase Bub1 pathway provides positional anchor leading to accumulation of H3T3ph on centromeres by prometaphase (Wang et al. 2011).

Thus, at the moment Haspin role in regulating mitosis in animal cells is thoughtfully investigated and rather well understood. However, recent studies in fungi and plants indicate additional Haspin functions in regulating cell division. Domain structure of proteins from Haspin group comprises a C-terminal catalytic domain and an N-terminal region of unknown structure that is highly divergent between Haspin homologues, which also suggests that Haspin can have different functions in various species. For instance, in yeast there is no evidence of H3T3ph, therefore Haspin function is inexplicable with animal model. Nevertheless, it was demonstrated that deletion of ALK1 and ALK2 Haspin paralogs causes the mislocalization of polarisome components. After a transient mitotic arrest, this led to an overly polarized actin distribution in the bud. Cell division was completed producing one anucleated and one binucleated daughter cell (Panigada et al. 2013). Interestingly, expression of *Arabidopsis thaliana* Haspin in *alk1Δalk2Δ* partially rescued the phenotype,

suggesting that Haspin function in cell polarity can conserved in other species (Panigada et al. 2013).

Haspin kinase in plant cells

In plants, Haspin kinase has been identified in *A. thaliana* (Kurihara et al. 2011; Ashtiyani et al. 2011). AtHaspin kinase domain showed rather low similarity (38%) to human Haspin, however, amino acid residues that bind ATP and Mg^{2+} are conserved. *In vitro* experiments have discovered that AtHaspin phosphorylates histone H3 at Thr3 and, surprisingly, also at Thr11 (Kurihara et al. 2011). Further analyzes indicated, that in the transgenic plant AtHaspin-GFP driven by the native promoter was expressed in tissues with high cell-division activity, such as meristems and during the embryo stage. Moreover, mutant with overexpression of the AtHaspin kinase domain had a phenotype with delayed root growth and decreased size of the root meristem (Kurihara et al. 2011); altered expression of the kinase induced pleiotropic phenotypes with defects in floral organs and vascular tissue, study also indicate that Haspin may contribute to embryonic patterning (Ashtiyani et al. 2011).

Aurora kinases role in regulating cell division in plants

Although Haspin role in regulating plant cell division remains unclear, other histone H3 kinase, Aurora kinases have a function in plant cell division. Three members from Aurora kinase family (AtAUR1, 2 and 3) were identified in *A. thaliana*; all three AtAURs phosphorylate histone H3 at Ser10 *in vitro,* AtAUR3 also phosphorylates histone H3 at Ser28 (Demidov et al. 2005; Kawabe et al. 2005; Kurihara et al. 2006). *In vivo,* both H3S10ph and H3S28ph localized in pericentromeric regions of chromosomes from prophase till anaphase. AtAUR3 kinase, a homolog of mammalian Aurora B, shows centromeric localization from pro- till metaphase, furthermore inhibition of AtAUR3 leads to delay in chromosome alignment and aberrant chromosome segregation (Kurihara et al. 2006; Kurihara et al. 2008; Eswaran et al. 2009). Nevertheless, the relation between Haspin kinase and Aurora family in plant cells has not been established yet. AtAUR1 and AtAUR2 are localized on the mitotic spindles during mitosis and have been found on the cell plate during cytokinesis. AtAUR1 and AtAUR2 most likely have redundant function in orientation of cell division plane through plant development (Van Damme et al. 2011).

Cytokinesis in plant cells: key aspects, regulation and questions.

Cytokinesis is distinctive feature in plant cell division due to the presence of the cell wall. Unlike contractile ring present in animals and yeasts, plant cell division is achieved by phragmoplast-guided cell plate formation and expansion towards cell

periphery (Figure 1; Jürgens 2005). Phragmoplast consists mostly of microtubule (MT) and actin filaments that form a highly dynamic structure, changes in which are essential for cytokinesis (Lee and Liu 2013; McMichael and Bednarek 2013). Although significance of phragmoplast for plant cell division is explicit, factors involved into regulation not fully understood yet. One of the described pathways is NACK-PQR cascade that governs cytokinesis through the regulation of phragmoplast MTs (Nishihama et al. 2002; Nishihama et al. 2001; Sasabe and Machida 2012). NACK-PQR cascade is downregulated by CDKs till metaphase, from anaphase interaction between mitotic kinesin NACK1 and MAPK kinase kinase NPK1 activates the cascade (Sasabe et al. 2011). All members from the cascade concentrate at the midzone of the phragmoplast during cytokinesis (Sasabe and Machida 2012), downstream MAPKs phosphorylate microtubule-associated protein 65 (MAP65), which in turn promotes MT turnover and cell plate expansion (Sasabe et al. 2006).

Although actin filaments are also a prominent part of phragmoplast array, actin role remains highly controversial. Disruption of actin with various drugs, such as profilin (Valster et al. 1997), bistheonellide A (Hoshino et al. 2003; Higaki et al. 2008), latrunculin B (Kojo et al. 2013) leads to delay in cell plate expansion and other aberrations in cytokinesis. In addition, there have been studies showing a link between actin function and motor protein myosin (Molchan et al. 2002). Moreover, recently myosin VIII together with actin was demonstrated to guide phragmoplast

expansion to the cortical division site in moss and tobacco (Wu and Bezanilla 2014). Thus, actin filaments appears to be essential for proper cytokinesis in plant cells, yet exact role and interactions remain unclear and call for further research. Although there is no evidence of Haspin kinase impact on cytokinesis in animal cells, data obtained in budding yeast suggests that Haspin kinase can be important for regulating cell division (Panigada et al. 2013).

5-Iodotubercidin as Haspin kinase inhibitor

5-Iodotubercidin (5-ITu) is a small molecule that inhibits Haspin kinase activity in mammalian cells, displacing CPC with Aurora B from centromeres. Chemical inhibition of Haspin also leaded to misaligned chromosomes followed by SAC override. (De Antoni et al. 2012; Wang et al. 2012). 5-ITu potently targets the ATP-binding site of human Haspin kinase (Eswaran et al. 2009). The selectivity of inhibitors for Haspin kinase has been investigated thoughtfully via *in vitro* assays and temperature-shift assays in several studies (Balzano et al. 2011; Fedorov et al. 2012; De Antoni et al. 2012). Experimental analyses showed that 5-ITu only weakly inhibited, or did not inhibit, the majority of mitotic kinases including Cdk1–Cyclin B, Aurora kinases, Nek2.

My PhD research aims to expand our current knowledge about Haspin kinase role in regulating plant cell division. Here, using 5-ITu, I examined function of Haspin kinase in regulating cell division in tobacco BY-2 cells. Inhibition of Haspin kinase leads to delay in chromosome alignment during prometa- /metaphase, however unlike animal cells does not affect SAC. Furthermore, 5-ITu treatment affects AUR3 localization and function on centromeres, providing evidence for a functional link between Haspin and AUR3 in plant cells. Additionally, inhibition of Haspin kinase leads to severe cytokinesis defect, which results in incomplete cell plate formation and two nuclei inside one cell. It provides evidence that Haspin kinase can have a dual role in regulation of cell division in plant cells.

RESULTS

5-ITu is a valid inhibitor of the plant Haspin kinase

Small molecule 5-ITu was reported as ATP-competitive inhibitor for human Haspin kinase (Eswaran et al. 2009). To verify that 5-ITu is a valid inhibitor for plant Haspin kinase, I performed both *in vitro* and *in vivo* assays. The *in vitro* kinase assay was conducted using purified GST-AtHaspin and GST-AtHaspin-KD (kinase-dead) with or without inhibitors. I screened a range of 5-ITu concentrations from 1 to 20 μM in this experiment. Phosphorylation of the GST-histone H3 tail at specific residues was detected by immunoblotting using anti-H3T3ph and anti-H3T11ph antibodies. As shown in previous studies (Kurihara et al. 2011), GST-AtHaspin phosphorylated H3 at Thr3 and Thr11, while GST-AtHaspin-KD showed no kinase activity. 5-ITu inhibited H3T3ph in a concentration-dependent manner, but it did not affect H3T11ph (Figure 5A). At 10 μM 5-ITu, H3T3ph signal cannot be detected visually. To confirm the specificity of 5-ITu against Haspin kinase, the same *in vitro* kinase assay was performed with purified GST-AtAUR3, which phosphorylates histone H3 at Ser10 and Ser28 (Kurihara et al. 2006). As expected, GST-AtAUR3 phosphorylated histone H3 at Ser10 which was detected with specific anti-H3S10ph antibody (Figure 5B). This phosphorylation was significantly reduced by hesperadin, an Aurora kinase inhibitor (Kurihara et al. 2006), at a final concentration of 10 μM. However, 5-ITu did not affect

H3S10 phosphorylation by GST-AtAUR3 *in vitro*, suggesting that 5-ITu shows high specificity for Haspin kinase in plant cells, same as for Haspin in mammalian cells.

To evaluate the effect of 5-ITu on Haspin *in vivo*, I used *Nicotiana tabacum* L. cv. Bright Yellow 2 (tobacco BY-2) cultured cells. BY-2 cells have high cell division activity and large chromosomes, making them an attractive object for research on plant cell division. I added 5-ITu at various concentrations to the BY-2 cell culture, and then incubated the cells for further 24 h on a rotary shaker at 26°C. After fixing cells, I performed indirect immunofluorescence analysis using an anti-H3T3ph antibody and DAPI as DNA marker. Mitotic stage was determined based on chromosome morphology, and phosphorylation of histone H3 at Thr3 in prometaphase cells was analyzed. At concentrations between 0.1 and 0.5 μM, 5-ITu caused a decrease in the immunofluorescence levels of H3T3ph. At 1 μM, H3Thr3ph could not be detected visually (Figure 6A). Quantitative data represents concentration-dependent reduction in the H3T3ph signal normalized against the DAPI signal (Figure 6B). At 1 μM 5-ITu, H3T3ph/DAPI ratio reduced till 0.12 ± 0.05 (average ± standard deviation) comparing to 0.49±0.16 in control (DMSO). Summarizing, an *in vivo* assay showed that in living cells, H3T3ph was more sensitive to inhibition; 1 μM 5-ITu caused significant inhibition compared with 10 μM in the *in vitro* kinase assay. Given that *in vivo* studies better reflect kinase behavior in living

cells, I considered the concentration of 1 μM 5-ITu sufficient to inhibit Haspin activity in BY-2 cells.

Identification and characterization of Haspin kinase in BY-2 cells.

To analyze the gene encoding Haspin kinase in BY-2 cells, I obtained a candidate Haspin kinase sequence from the cDNA library of BY-2 cells created by RNA-Seq. I cloned the candidate Haspin cDNA from the cDNA library of 3-day-old BY-2 cells using specific primers (Table 2) designed based on the RNA-Seq data (kindly provided by Dr. T. Suzuki, Chubu University). The identified cDNA, designated as *NtHaspin* (*N. tabacum* Haspin-related gene; accession no. LC052296), was 1,902-bp long and encoded a protein of 633 amino acid residues. The amino acid sequence of NtHaspin showed 68% and 39% similarities with *A. thaliana* and human Haspins in the kinase domain, respectively (Figure 7). Similar to AtHaspin, NtHaspin contained the conserved residues that act as ATP- and Mg^{2+} ion-binding sites and are important for histone H3 phosphorylation in the catalytic cleft (Kurihara et al. 2011).

To observe the subcellular localization of NtHaspin throughout the cell cycle, I made a transgenic BY-2 line expressing NtHaspin-mClover and tdTomato-CenH3, a marker for the centromeric region of chromosomes (Figure 8). During interphase, NtHaspin signal was observed in the cytoplasm. After nuclear envelope breakdown, it

immediately invaded the nuclear region. During prometaphase and metaphase, NtHaspin was concentrated over the mitotic spindle, and a stronger signal was observed on chromosomes aligned at the equatorial plate. During cytokinesis, starting from late telophase, NtHaspin co-localized to the phragmoplast. Following phragmoplast expansion, NtHaspin moved towards the cell periphery. This localization pattern of NtHaspin was similar to that of AtHaspin (Kurihara et al. 2011). 5-ITu treatment did not affect NtHaspin localization (Figure 8), suggesting that the kinase activity of NtHaspin is not required for its localization.

Phosphorylation of histone H3 through the cell cycle in BY-2 cells.

I conducted another *in vivo* assay to check H3T3ph and H3T11ph throughout the cell cycle with or without 5-ITu treatment, using anti-H3S28ph antibodies as a negative control for immunostaining (Figure 9, 10). BY-2 cells treated with DMSO had strong signals from both H3T3ph (Figure 9) and H3T11ph (Figure 10), distributed evenly on the chromosomes during prometa- and metaphase; weak signal was sometimes observed in anaphase, no phosphorylation at Thr3 or Thr11 was detected in telophase to interphase cells (Figure 9, 10 DMSO; H3T3ph, H3T11ph). H3S28ph was observed as dot-like signal co-localized to centromeric region from prophase until anaphase. During anaphase H3S28ph was evenly distributed on chromosomes and disappeared by telophase (Figure 9, 10 DMSO; H3S28ph) . As expected,

treatment with 1 μM 5-ITu strongly decreased H3T3ph, and, surprisingly, H3T11ph and H3S28ph as well (Figure 9, 10 5-ITu). Since lower concentrations of 5-ITu were required to inhibit H3T3ph *in vivo* than *in vitro*, I suggest that H3T11ph is also more sensitive to inhibition by 5-ITu *in vivo*. The behavior and interactions of Haspin kinase are poorly understood; therefore, some unknown factors in living cells, such as changes in protein conformation, might make it more sensitive to 5-ITu. However, H3S28 is a substrate of AUR3 kinase, which phosphorylation is consistent with kinase concentration in centromeric area of chromosomes. Phosphorylation activity of AtAUR3 kinase was not affected by 5-ITu *in vitro*, even at higher concentrations of inhibitor (Figure 5B). The same result was reported previously for mammalian cells (Balzano et al. 2011). Therefore direct inhibition of AUR3 is not a likely explanation for decrease in H3S28ph signal under 5-ITu treatment. Previously in mammalian cells, it was shown that Haspin function is essential for centromeric localization of Aurora B, a homolog of plant AUR3 kinase (De Antoni et al. 2012; Wang et al. 2012). Therefore I suggested that similar pattern can conserved in plant cells and analyzed localization of NtAUR3 in BY-2 cells with inhibited Haspin kinase.

Localization of NtAUR3 on centromeres is associated with NtHaspin function

To determine whether the localization of AUR3 was affected by 5-ITu treatment, I performed indirect immunofluorescence analyses using anti-H3T3ph and anti-H3S28ph antibodies in BY-2 cells expressing NtAUR3 (accession no. LC052299)-mClover (Figure 11). In order to enhance signal from NtAUR3-mClover, anti-GFP antibody were used for immunostaining as well. In control cells NtAUR3 co-localized to centromeric region of chromosomes, consistent with its phosphorylation activity at H3S28 (Figure 11, DMSO). However, cells incubated with 5-ITu showed weak or no NtAUR3-mClover signal in the centromeric region and no H3S28 phosphorylation was detected (Figure 11, 5-ITu). To rule out the possibility that direct inhibition of NtAUR3-mClover caused kinase mislocalization, I tested the Aurora kinase inhibitor hesperadin at a final concentration of 5 μM. Unlike 5-ITu effect, the cells treated with hesperadin retained the NtAUR3-mClover signal on centromeres, although H3S28 phosphorylation was also inhibited (Figure 11, Hesperadin). Live-cell imaging of BY-2 cells expressing NtAUR3-mClover confirmed that 5-ITu displaced NtAUR3-mClover from the centromeres (Figure 12). These results suggested that under 5-ITu treatment, decrease in H3S28ph signal caused by NtAUR3 displacement from centromeres, rather than direct inhibition of the NtAUR3 kinase function. Furthermore, I presume that NtAUR3 localization in the centromeric region of chromosomes during mitosis is associated with NtHaspin kinase function.

5-ITu treatment increases duration of mitosis and alters chromosome alignment

As shown in Figure 13, 5-ITu treatment severely affected cell division in BY-2 cells. To analyze cell growth and cell cycle progression, I calculated the mitotic index after a 24-h incubation with 5-ITu at a broad range of concentrations from 0.1 μM to 10 μM. BY-2 cells showed a concentration-dependent decrease in the mitotic index. The minimum mitotic index was 0.92 ± 0.28% (*n* = 4 experiments) at 1 μM 5-ITu (Figure 13A). This result indicated that 5-ITu could prevent BY-2 cells from entering mitosis. Hence, 5-ITu slowed the growth rate of the cell culture. Quantitative data obtained from live-cell imaging showed that mitosis took longer under inhibitor treatment (Figure 13B). Live-cell imaging was conducted in BY-2 line expressing tdTomato-CenH3 for 24 hr. Time-lapse was taken every 5 minutes and mitotic stage was determined based on chromosome behavior. In control cells treated with DMSO, the average length of mitosis, from pro- till telophase, was 60 ± 3 min (*n* = 67). However, average mitosis length grew up to 113 ± 7 min (*n* = 13) in BY-2 cells incubated with 1 μM 5-ITu.

Live-cell imaging in BY-2 cells expressing tdTomato-CenH3 revealed that under 5-ITu treatment, the prolonged duration of mitosis was because of a delay in chromosome alignment during prometa/metaphase (Figure 13C, lagging chromosomes indicated with white arrowheads). However, the spindle assembly

checkpoint was not disturbed, thus metaphase/anaphase transition occurred no sooner than all chromosomes were aligned at the equatorial plate (Figure 13C). Together, these results showed that BY-2 cells in which Haspin kinase was inhibited by 5-ITu remained in the prometaphase/metaphase stages for longer time, which increased the duration of mitosis.

Inhibition of Haspin kinase causes cytokinesis defect in BY-2 cells

In the live-cell imaging experiments (Figure 8, Figure 12), I frequently observed bizarre cytokinesis in cells treated with 5-ITu. It led to investigation of the 5-ITu effect on the final stage of cell division. For this purpose, I used a transgenic BY-2 line expressing GFP-KNOLLE (syntaxin KNOLLE is a specific cell plate marker;(Lauber et al. 1997) to observe the cytokinesis process. As expected, control cells treated with DMSO showed normal cytokinesis, in which cell plate initiated in late telophase between two nuclei and expanded centrifugally until fused with the lateral cell walls (Figure 14, DMSO). However, BY-2 cells treated with 5-ITu showed cytokinesis defect (Figure 14, 5-ITu). The initiation of the cell plate also started at late telophase, and the cell plate expanded and usually fused with one lateral cell wall. At this point, the process stopped and the cell plate did not continue expansion to the other side of the cell. Even after 2 h, there was no progress in cytokinesis and the cells still had a half-formed cell plate. Another characteristic of the cytokinesis defect

was the blocked movement of nuclei, which remained attached to the half-formed cell plate (Figure 14, DMSO, 5-ITu nuclei position is marked with yellow circles). Quantitative data obtained from time-lapse live-imaging in GFP-KNOLLE showed significant difference ($p < 0.0001$ by two-tailed Fisher's exact test) between cytokinesis defect frequency in BY-2 cell culture treated with DMSO (control) and 1 μM 5-ITu (Table 1). Summarizing, 5-ITu treatment caused severe cytokinesis defect in BY-2 cell culture, leading to binuclear cells with half-formed cell plate. Up to current knowledge, 5-ITu does not have same effect on mammalian cells, indicating that 5-ITu inhibits some cytokinesis pathway unique for plant cells. However, the mechanism underlying cytokinesis defect remained unclear and called for further screening. Inhibition of vesicle transport or fusion can inhibit cytokinesis, therefore we checked accumulation of callose by aniline blue staining (Figure 15). Both control (DMSO) and 5-ITu treated cells show bright blue fluorescence in the cell plate. It indicates that vesicle traffic to the cell plate was not affected by 5-ITu treatment, which is also supported by normal distribution of KNOLLE.

Since 5-ITu treatment caused cytokinesis defect, I also checked cell culture ploidy. After 24 hr treatment with DMSO, 5-ITu or Hesperadin, BY-2 cells were collected and analyzed using cell sorter (Figure 16). I observed two peaks: first peak showing percentage of diploid cells second peak that shows tetraploid cells. As

expected, 5-ITu treatment increased amount of the 4C nuclei from 19.3% in DMSO sample to 36.4% in 5-ITu sample.

Other members of the Aurora family, AUR1 and AUR2, were previously described as kinases important for cytokinesis in plants (Van Damme et al. 2011), however their substrates and interactions in cytokinesis pathways remain unknown. I checked the localization of NtAUR1 (accession no. LC052297)-mClover and NtAUR2 (accession no. LC052298)-mClover in BY-2 cells throughout the cell cycle under 5-ITu treatment. In control cells treated with DMSO, both NtAUR1 and NtAUR2 were concentrated on the nuclear membrane prior to nuclear envelope breakdown and on the mitotic spindle during mitosis. Later, they were localized in the phragmoplast region (Figure 17, 18, DMSO). These localization patterns were similar to those of AtAUR1 and AtAUR2 (Demidov et al. 2005; Kawabe et al. 2005). Treatment with 5-ITu did not affect the distribution of NtAUR1 and NtAUR2 during cell division (Figure 17,18, 5-ITu). These results suggested that Haspin kinase activity is not required for the functions of NtAUR1/2 in cytokinesis.

The NACK-PQR pathway, a NACK1 kinesin-like protein and mitogen activated protein kinase (MAPK) cascade, is a key regulator of plant cytokinesis. This pathway regulates the assembly of phragmoplast MTs (Nishihama et al. 2001; Nishihama et al. 2002; Soyano et al. 2003). I checked whether 5-ITu affects NACK1 distribution in BY-2 line expressing GFP-NACK1. Live-imaging data (Figure 19, DMSO) shows that

in control cells NACK1 signal is restricted to the expanding cell plate during cytokinesis. In 5-ITu-treated cells NACK1 was observed on the cell plate for longer time then in control (Figure 19, 5-ITu), however, as expected, cell plate did not complete expansion. There are several members involved in the NACK-PQR cascade, however, up to current knowledge the most downstream is NtMAP65-1, which phosphorylation stimulates phragmoplast expansion in tobacco (Sasabe et al. 2006). To determine the effect of 5-ITu on the NACK-PQR pathway, I performed indirect immunofluorescence staining using anti-NtMAP65-1, anti-NtMAP65-1ph, and anti-α-tubulin antibodies. In control cells, both MAP65-1 and MAP65-1ph were co-localized with MTs in the phragmoplast (Figure 20, DMSO). 5-ITu treatment did not had strong effect the localization or the phosphorylation of MAP65-1 (Figure 20, 5-ITu). These data suggested that NtHaspin kinase inhibition with 5-ITu did not affect the NACK-PQR pathway.

5-ITu disturbs actin dynamics during cytokinesis

Expansion of the cell plate is guided by MT and actin filaments array called phragmoplast, which dynamic changes are essential for proper cytokinesis (Lee and Liu 2013). Actin filaments are involved in cytokinesis, however their role remain speculative. A Haspin kinase knockout mutant of yeast showed altered actin distribution during mitosis (Panigada et al. 2013). To explore the role of the

cytoskeleton in the cytokinesis defects caused by 5-ITu, I tested the effect of 5-ITu on BY-2 cells expressing three markers: GFP-α-tubulin (microtubules), tdTomato-CenH3 (centromeres), and Lifeact-mTurquoise2 (F-actin). In control cells treated with DMSO, MTs formed the mitotic spindle, and both MTs and actin filaments were concentrated around the cell plate at the start of cytokinesis. As the cell plate expanded, cytoskeleton elements depolymerized in the center and repolymerized along the edge of the growing cell plate (Figure 21, DMSO). In the cells treated with 5-ITu, the MT distribution was the similar to the control, however there was low to none concentration of actin filaments at the cell plate (Figure 21, 5-ITu). Actin role in cytokinesis is not clear yet, therefore this data provides interesting insights on the importance of actin filaments in plant cell division and requires further investigation.

Next, I explored whether changes in actin dynamics alone could explain the cytokinesis defect. BY-2 cells expressing GFP-KNOLLE were treated with 2 μM Latrunculin B, a drug that disrupts the actin cytoskeleton (Wakatsuki et al. 2001). Disruption of actin filaments led to a significant delay in cytokinesis (Figure 22, Latrunculin B). Corresponding with the results published by other groups (Kojo et al. 2013), cell plate expansion was aberrant and tilted cell planes were frequently observed in actin-disrupted BY-2 cells. However, the late cell plate expansion was not completely blocked by Latrunculin B, as opposed to the arrested cell plate expansion caused by 5-ITu treatment. Previous studies of drugs other than Latrunculin B have

not been able to demonstrate that the absence of actin filaments alone leads to severe cytokinesis defects (Nishimura et al. 2003). Quantitative data of cell plate expansion rates is presented in Figure 23. Comparing to Latrunculin B, 5-ITu treatment has stronger effect of the cell plate expansion. Average cell plate size is also smallest in 5-ITu sample, which reflects cytokinesis defect and complete inhibition of the cell plate expansion, which occurred in some cells. Taken together, these results indicate that other factors beside actin are involved in the cytokinesis defect caused by 5-ITu.

DISCUSSION

To the best of my knowledge, there have been very few studies reporting Haspin kinase function in plant cells. Haspin kinase in plants was first discovered in *A. thaliana* (Kurihara et al. 2011; Ashtiyani et al. 2011). Haspin function as H3T3 kinase was conserved, furthermore new substrate H3T11 was identified (Kurihara et al. 2011). Haspin expression pattern and mutant analysis indicates potential function in embryo development (Ashtiyani et al. 2011). Nevertheless, Haspin role in regulating cell division and interaction network in plants remained ambiguous.

Based on the results of this study, I have expanded the hypothesis about the role of Haspin kinase in regulating cell division in plants (Figure 24). Experiments with the chemical inhibitor 5-ITu revealed that Haspin kinase is likely to contribute to chromosome alignment during prometaphase/metaphase, and can be involved in the late cell plate expansion during cytokinesis.

Relationship between Haspin and AUR3 kinase in plant cells.

I showed that 5-ITu inhibits H3T3ph both *in vitro* and *in vivo*; however, H3T11ph was inhibited by 5-ITu only *in vivo*. Compared with the *in vitro* assay, the *in vivo* assay in BY-2 cells was more sensitive to inhibitor treatment. Therefore, it is possible that higher concentrations of 5-ITu could inhibit H3T11ph *in vitro.* I also

demonstrated that 1 μM 5-ITu was sufficient to inhibit Haspin kinase function in BY-2 cells, compared with 10 μM used in experiments on mammalian cells (De Antoni et al. 2012; Wang et al. 2012). Based on these results, 5-ITu is an effective inhibitor of the plant Haspin kinase. Several studies have analyzed 5-ITu selectivity in mammalian cells (Balzano et al. 2011; De Antoni et al. 2012); however, corresponding experiments have not been conducted for plant kinases so far. Here, I tested the specificity of 5-ITu for the AUR3 kinase, as the most likely candidate for the Haspin regulatory pathway. In plant cells, AUR3 kinase has two known substrates, H3S10 and H3S28; both are phosphorylated in the pericentromeric region of chromosomes from prophase until metaphase (Kawabe et al. 2005). In the *in vitro* assay, 5-ITu did not inhibit H3S10ph even at high concentrations, although the AUR3-specific inhibitor hesperadin significantly reduced the phosphorylation level (Figure 5B). However, in the *in vivo* assay, BY-2 cells treated with 5-ITu showed no detectable H3S28ph, consistent with the displacement of NtAUR3 from the centromeric region. Hesperadin treatment reduced H3S28ph, yet it did not affect NtAUR3 localization. These data showed that *in vivo* 5-ITu treatment caused NtAUR3 mislocalization, which affected NtAUR3 function at the pericentromeric region of chromosomes. Since *in vitro* it was demonstrated that 5-ITu inhibits Haspin kinase, but not NtAUR3, I suggest that NtAUR3 displacement from centromeres is likely caused by inhibition of Haspin function. Therefore Haspin function in plant cells is important for centromeric

localization and function of AUR3 kinase during mitosis. Although Aurora kinase family is diversified in plants and animals, Aurora B kinase from mammalian cells and AUR3 kinase from plant cells share localization areas during mitosis and at least some functions, including centromeric phosphorylation of H3S10 and H3S28. It indicates that relationship between Aurora kinase family and Haspin kinase is conserved.

Nevertheless, it should be noted that histone H3 phosphorylation pattern differs between in mammalian and plant cells. In animal cells, H3T3 is phosphorylated on centromeres during prometaphase and metaphase (Dai et al. 2005; Markaki et al. 2009), but in plants, the H3T3ph signal is evenly distributed on chromosomes (Kurihara et al. 2011). I presume that Haspin kinase function is essential for the centromeric localization of AUR3 in plant cells, but whether it is associated with H3T3ph or depends upon other interactions is open for further investigation.

The cell division of BY-2 cells treated with 5-ITu showed several deviations from normal cell division. 5-ITu treatment significantly reduced mitotic index in the cell culture, however the reasons that prevent BY-2 cells from entering mitosis are unknown. Similar effect 5-ITu has on HeLa cells (De Antoni et al. 2012). Furthermore, mitosis progression from prophase till telophase was significantly longer in BY-2 cells treated with 5-ITu. Live-imaging of labeled centromeres revealed that there was a

delay in chromosome alignment along the equatorial plane in prometa/metaphase. Previously, Haspin downregulation by RNAi was shown to cause chromosome misalignment and partial loss of chromatid cohesion (Dai et al. 2006; Dai et al. 2009). Treatment with 5-ITu had a similar effect in HeLa cells (De Antoni et al. 2012; Wang et al. 2012). However, in BY-2 cells, 5-ITu did not cause spindle assembly checkpoint override or defects during anaphase, possibly due to lower concentrations of 5-ITu used in my experiments. Direct inhibition of AUR3 with hesperadin was also shown to cause delay in chromosome alignment in plant cells (Kurihara et al. 2008), indicating that the centromeric function of AUR3 is important for prometa/metaphase progression. These findings suggest that Haspin and Aurora are involved in the same pathway, where Haspin is upstream and provides spatial information, recruiting AUR3 to centromeres. Therefore, 5-ITu inhibition of Haspin function also downregulates AUR3, leading to the delay in chromosome alignment.

Haspin kinase has a potential function in cytokinesis regulation.

Here, I also describe previously uncharacterized cytokinesis defect in BY-2 cells treated with 5-ITu (Fig. 14). In many BY-2 cells treated with 5-ITu, late cell plate expansion was completely inhibited, and the movement of nuclei was blocked. Up to best of my knowledge, neither 5-ITu treatment nor Haspin downregulation by RNAi resulted in cytokinesis abnormalities in animal cells. Therefore, I suggest that 5-ITu

affects a regulatory pathway that is unique to plants. I checked several elements, known to be involved in cytokinesis in plant cytokinesis: accumulation of callose in the cell plate, distribution of syntaxin KNOLLE, AUR1 and AUR2 kinases, the NACK-PQR pathway, MT and actin dynamics. Cytokinesis defect can be caused by interference with vesicle transport (Verma 2001), however aniline blue staining revealed that 5-ITu does not change accumulation of callose in the cell plate (Figure 15). Localization of syntaxin KNOLLE also remained the same. These results led to conclusion that 5-ITu treatment does not affect vesicle trafficking, and cytokinesis defect occurs due to other reasons.

Other members from Aurora family, AtAUR1 and AtAUR2, previously have been demonstrated to have function in cytokinesis (Van Damme et al. 2011). Localization of NtAUR1 and NtAUR2 was not affected by 5-ITu treatment. The role of the AUR1/2 pathway and its targets during cytokinesis are currently unknown; however, the involvement of AUR1/2 in the 5-ITu phenotype does not seem likely. The *Arabidopsis* double mutant *aur1aur2* showed defective cell division orientation throughout development, but cell plate expansion was not severely unaffected (Van Damme et al. 2011).

The NACK-PQR pathway, which regulates MT turnover and phragmoplast expansion, was also unaffected by 5-ITu treatment. Although I have not tested all of the components of the NACK-PQR pathway in this study, distribution of kinesin

NACK1 remained same. The phosphorylation and localization of the most downstream member, MT-associated protein NtMAP65-1, also remained intact under 5-ITu treatment. Surprisingly, 5-ITu treatment had no visual impact on MT dynamics, even though severe cytokinesis defects are usually associated with disturbed MT dynamics (Lee and Liu 2013). Thus, I consider that NtHaspin has no relationship with these pathways.

The phragmoplast consists of both MT and actin filaments, yet the role of actin in cytokinesis remains ambiguous. Here, I demonstrated that one characteristic of the 5-ITu-induced cytokinesis defect was impaired actin colocalization to the cell plate (Figure 21). The reasons for the disrupted actin dynamics remain unclear; however, studies on yeast have demonstrated a link between Haspin kinase and actin distribution; that is, the Haspin kinase knockout mutant showed abnormal actin distribution during cytokinesis, which resulted in binuclear cells (Panigada et al. 2013). Furthermore, Haspin was shown to localize specifically in the region enriched with actin in mouse oocytes (Nguyen et al. 2014). Taken together, these observations and results suggest that the relationship between Haspin and actin can be conserved in various species. Recently, the actomyosin-mechanism of phragmoplast guidance was reported in moss (Wu and Bezanilla 2014). Myosin VIII was shown to accumulate at MT plus ends and at the cortical division zone, while F-actin connected the phragmoplast edge to the periphery membrane. It was suggested that Myosin VIII

uses actin bridges to connect the phragmoplast MT to the cortical division site (Wu and Bezanilla 2014). Additionally, a phenotype very similar to that under 5-ITu treatment was observed in *Tradescantia* stamen hair cells treated with an inhibitor of the myosin light-chain kinase. These cells showed inefficient cytokinesis, up to complete inhibition late cell plate expansion (Molchan et al. 2002). Analysis of cell plate expansion rates between control, 5-ITu treatment and Latrunculin B indicates that other factors, beside actin, are involved into cytokinesis defect, one of the potential candidates for future investigation are myosin proteins.

In summary, treatment of BY-2 cells with a Haspin kinase inhibitor led to severe cytokinesis defect, potentially caused by disturbed actomyosin-guided phragmoplast expansion. Actomyosin complex and distribution of polarity factors, such as formins, should be analyzed in further studies to establish their relationships with Haspin kinase.

Summary

Based on our data, I propose the following hypothesis for Haspin function in regulating cell division in plant cells (Figure 24): during prometa/metaphase, Haspin kinase function is necessary for the centromeric localization of AUR3. One possibility is that H3T3ph is a conserved mechanism for recruitment of AUR3 to centromeres.

As cell division progresses, Haspin appears to be involved in different pathways regulating late cell plate expansion. Inhibition of Haspin led to cytokinesis defect and the formation of binuclear cells. Other factors involved into this pathway are unknown (Figure 24, Factor X); however, I suggest actomyosin as a prospective downstream candidate in the Haspin cascade.

MATERIALS AND METHODS

Tobacco BY-2 cell culture

The tobacco BY-2 (*Nicotiana tabacum* L. cv. Bright Yellow 2) cell culture was maintained as described by Nagata et al. (1992). BY-2 cells were cultured in modified Linsmaier and Skoog medium on a rotary shaker at 100 r.p.m. at 26°C in the dark. GFP-KNOLLE and GFP-NACK1 lines were kindly provided by Dr. M. Ito (Nagoya University) and Dr. M. Sasabe (Hirosaki University), respectively.

Cloning and transformation

I used BLAST search to find sequences for Haspin and Aurora kinase candidates using RNA-seq database for 2, 5, and 7 day-old BY-2 cells (kindly provided by Dr. T. Suzuki, Chubu University). For vector construction, mRNAs were isolated from 3-day-old BY-2 cells using Dynabeads Oligo $(dT)_{25}$ (Invitrogen) and cDNAs were synthesized by SuperScript III reverse transcriptase (Invitrogen). The PCR primers used in this study are listed in Table 2. The cDNAs of *NtHaspin*, *NtAUR1*, *NtAUR2*, and *NtAUR*3 were subcloned into the spUC-mClover vector, which contains the CaMV 35S promoter as described by Kurihara et al. (2011). The resulting 35Spro::cDNA-mClover was inserted into the binary vector pPZP211 (Hajdukiewicz et al. 1994). The Lifeact-mTurquiose2 sequence, which was obtained from Addgene

(No. 36201), was also inserted into the pPZP211 vector. The binary vectors were introduced into *Agrobacterium tumefaciens* strain EHA105. Transformation of BY-2 cells and BY-GTRC (expressing GFP–α-tubulin–RFP–CenH3) cells (Kurihara et al. 2008) was performed as described by Kurihara et al. (2011).

In vitro kinase assay

The *in vitro* kinase assay was performed with purified GST-AtHaspin or GST-AtHaspin-KD or GST-AtAUR3 and GST-histone H3 tail as the substrate, as described previously (Kurihara et al. 2006; Kurihara et al. 2011). In brief, inhibitor (5-ITu or hesperadin) was added to the reaction mixture at the desired concentration before adding ATP. Phosphorylated histone H3 was determined by immunoblotting using 1:2000 dilutions of rabbit polyclonal antibodies against H3T3ph and H3T11ph (Upstate Biotechnology) and rabbit polyclonal antibodies against H3S10ph (Millipore). Immunoreactive proteins on the PVDF membranes were detected with a 1:20000 dilution of peroxidase-conjugated goat anti-rabbit IgG antibody (KPL) and Immobilon Western Chemiluminescent HRP Substrate (Millipore) using a luminescent image analyzer (LAS-4000 mini; GE Healthcare).

Fluorescent microscopy

I essentially followed the inhibitor treatment and immunostaining procedures described previously (Kurihara et al. 2006). For the inhibitor treatment, 2-day-old BY-2 cells were incubated with the desired concentration of inhibitor 24 hr before fixation. Cells were fixed using 4% (w/v) PFA in PBS and placed on coverslips pre-treated with poly-L-lysine. Cell walls were briefly digested with an enzyme mixture consisting of 2% (w/v) cellulose and 0.5% (w/v) pectolyase in PBS. Next, the cells were permeabilized using 0.5% Triton X-100 in PBS, then blocked with 4% (w/v) BSA in PBS and incubated with appropriate primary antibodies diluted in PBS overnight at 4°C. I used a 1:500 dilution of rabbit polyclonal antibodies to H3T3ph and H3T11ph (Upstate Biotechnology), a 1:500 dilution of rat monoclonal antibodies to H3S28ph (Abcam), a 1:500 dilution of goat polyclonal antibodies to GFP (Abcam), a 1:200 dilution of mouse monoclonal antibodies to α-tubulin (Sigma), and 1:100 dilutions of rabbit polyclonal antibodies to NtMAP65-1α (anti-MAP65) and Thr-579-phosphorylated NtMAP65-1α (anti-pT579MAP65) (Sasabe et al. 2006). After the primary antibody reaction, cells were washed twice in PBS and then incubated with the respective secondary antibodies at the following dilutions in PBS for 3 h at room temperature: 1:200 dilutions of Alexa 594-conjugated goat anti-rabbit IgG antibodies, Alexa 488-conjugated goat anti-rat IgG antibodies, and Alexa 488-conjugated goat anti-mouse antibody (Invitrogen Molecular Probes). Cells were washed twice in PBS,

and then mounted with DAPI solution (2 μg/ml). Images were acquired with an Olympus BX-51 fluorescence microscope.

For aniline blue staining, I followed protocol described by Nishihama et al. (2001) with the following modifications: cells were fixed with 0.1% glutaraldehyde in 50 mM sodium phosphate buffer (pH 7.5) for 1 h, washed three times in 50 mM Na_2HPO_4. Cells were stained with 0.1% aniline blue in 50 mM Na_2HPO_4.

Flow Cytometry

Three-day-old BY-2 culture was treated with 1 μM 5-ITu, 5 μM hesperadin or DMSO (control). After 24 hr cells were collected and frozen in liquid N_2. Frozen pellets were used for nuclei extraction using CyStain UV Precise P kit (Partec, Germany) following manufacture's instructions. DNA content was analyzed using a cell sorter (SH800; Sony, Japan).

Live-cell imaging

BY-2 cells expressing NtHaspin-mClover, GFP-KNOLLE and GFP-NACK1 were observed using a Nipkow disk-type confocal microscope (CV1000; Yokogawa Electric). BY-2 cells expressing NtAUR1,2,3-mClover or BY-2 cells expressing GFP-α-tubulin, tdTomato-CenH3, and Lifeact-mTurquoise2 were observed using a

fluorescence microscope (IX-83; Olympus) equipped with a Nipkow disk confocal unit (CSU-W1; Yokogawa Electric). For inhibitor treatments, cells were incubated with the desired concentration of inhibitor for 1 hr before observation. Images were processed using ImageJ software (http://rsbIb.nih.gov/ij/index.html) and Adobe Photoshop CS6 (Adobe Systems, Inc.).

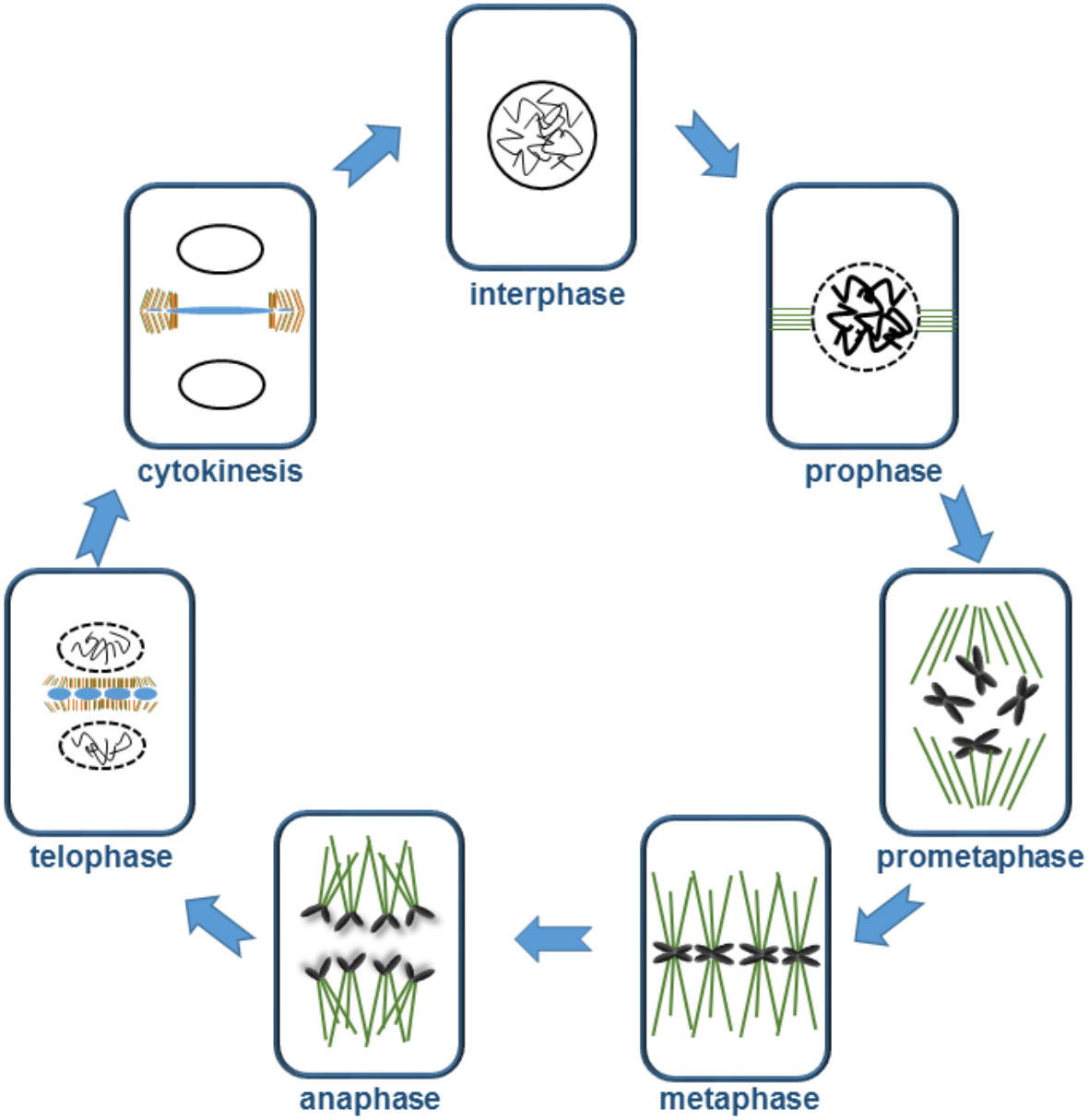

Figure 1. Schematic representation of plant cell division

Plant cell division is distinguished by preprophase band mapping future division site early in mitosis. Cytokinesis occurs via phragmoplast, an array of MT and actin filaments that guide vesicle traffic and cell plant expansion.

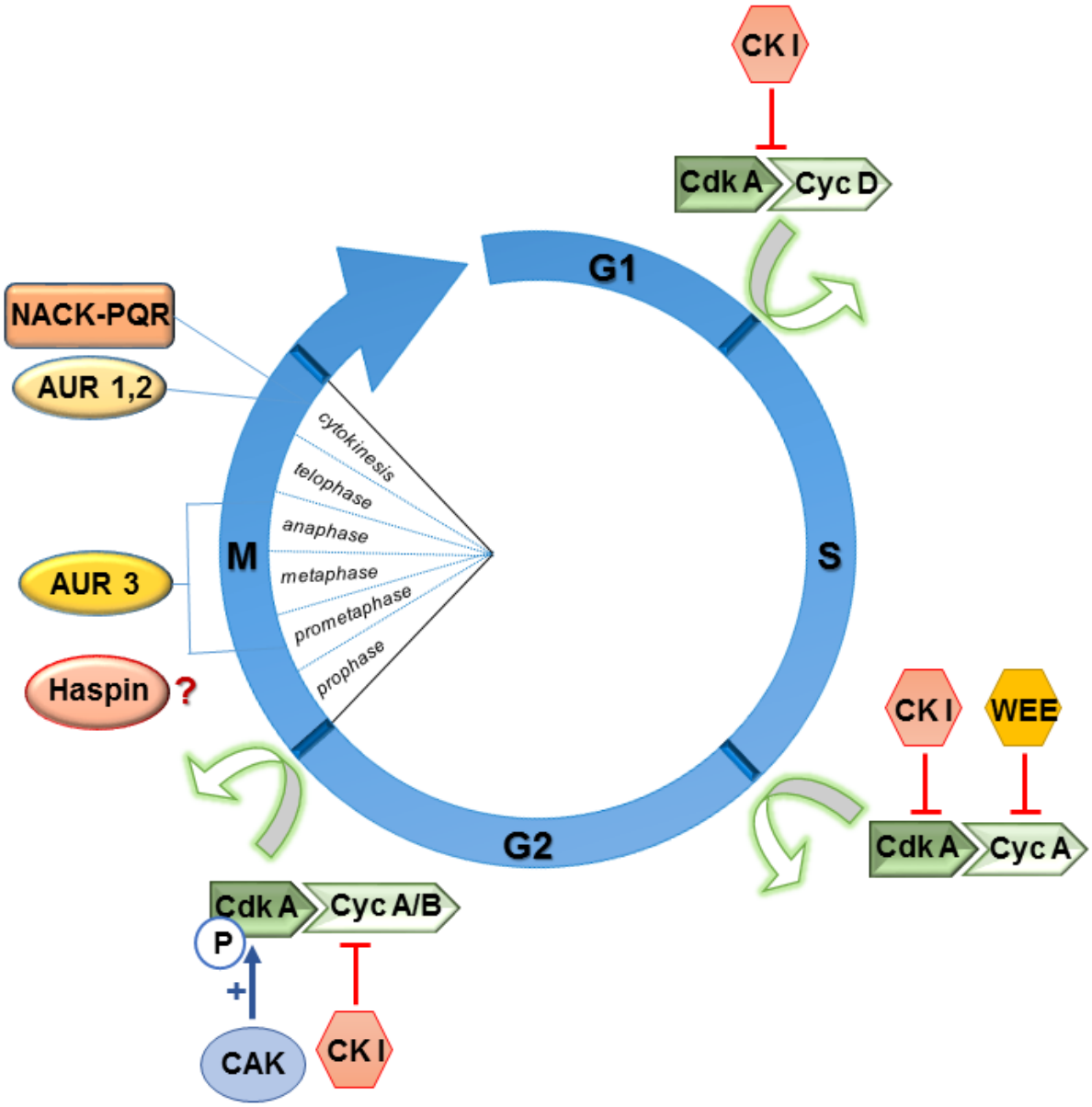

Figure 2. Regulation of cell cycle transitions and cell division in plants

Cell cycle progression requires formation activation and following inactivation of cyclin-dependent protein kinases (CDKs). CDKs bind to various cyclins, responsible for differential activation of kinase through the cell cycle. CDKs complexes are kept inactivate by phosphorylation by Wee1 kinase or binding to CKIs proteins. Phosphorylation of CdkA-CycB by CAK kinase triggers G2 to M transition. Refer to the text for details of cell division regulation.

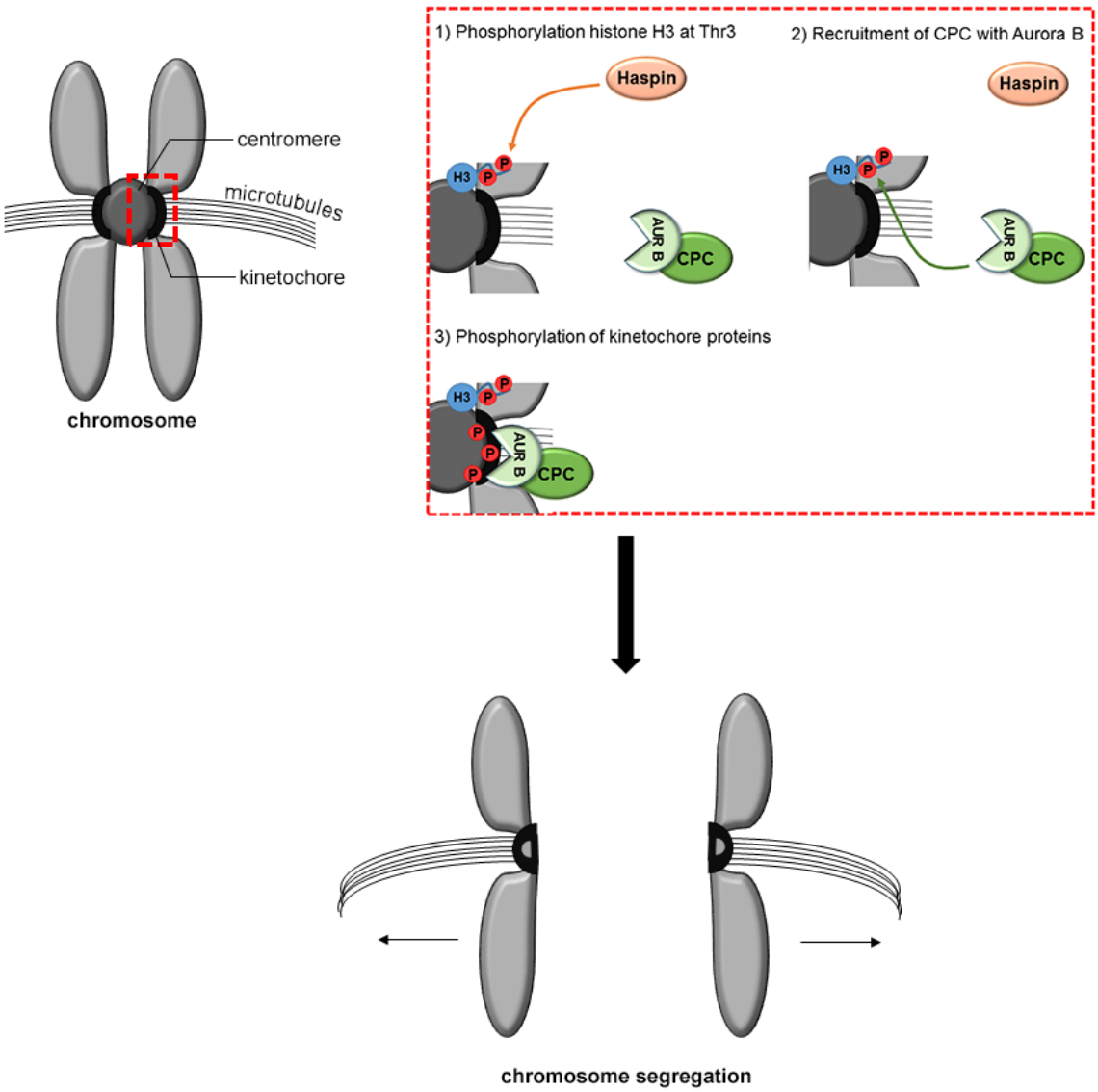

Figure 3. Haspin role in regulating mitosis in animal cells

A cascade of molecular interactions and phosphorylation events occur at the pericentromeric regions during chromosome alignment and segregation. The inner kinetochores at the pericentromeric regions are attached to the mitotic spindle (microtubules). Haspin and CPC with Aurora B involved in these events, which control the equal chromosome segregation (Musacchio 2010).

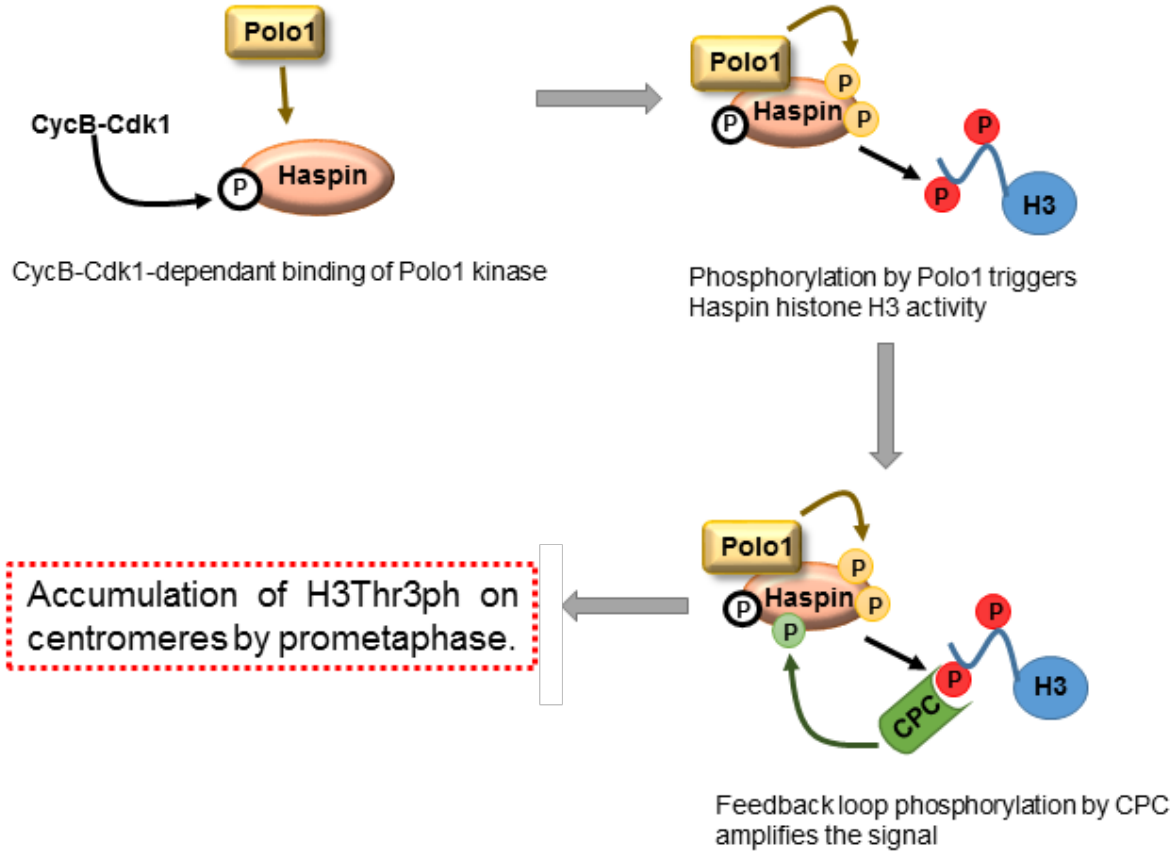

Figure 4. Regulation of Haspin kinase and triggering H3T3ph modification.

See text for details (Zhou et al. 2014)

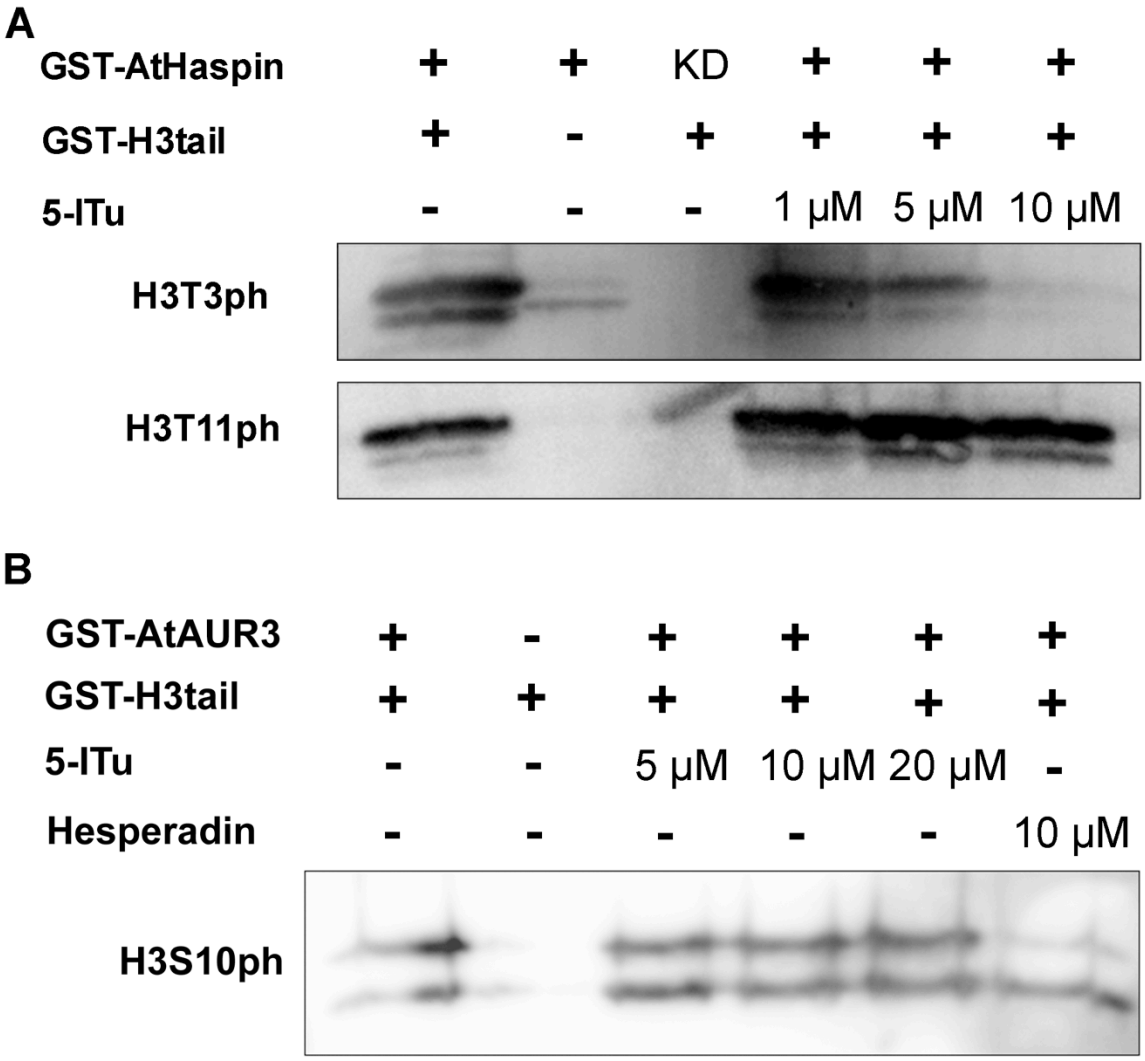

Figure 5. 5-ITu inhibits Haspin kinase function *in vitro*

In vitro kinase assay followed by Western Blotting. GST-AtHaspin, GST-AtHaspin-KD (A), and GST-AtAUR3 (B) were incubated with GST-H3 tail. Negative control: GST-H3 tail only. 5-ITu was added at a final concentration of 1, 5, 10 or 20 µM; hesperadin was added at final concentration of 10 µM. Phosphorylated GST-H3 tail was immunostained using anti-H3T3ph, anti-H3T11ph (A), and anti-H3S10ph (B) antibodies.

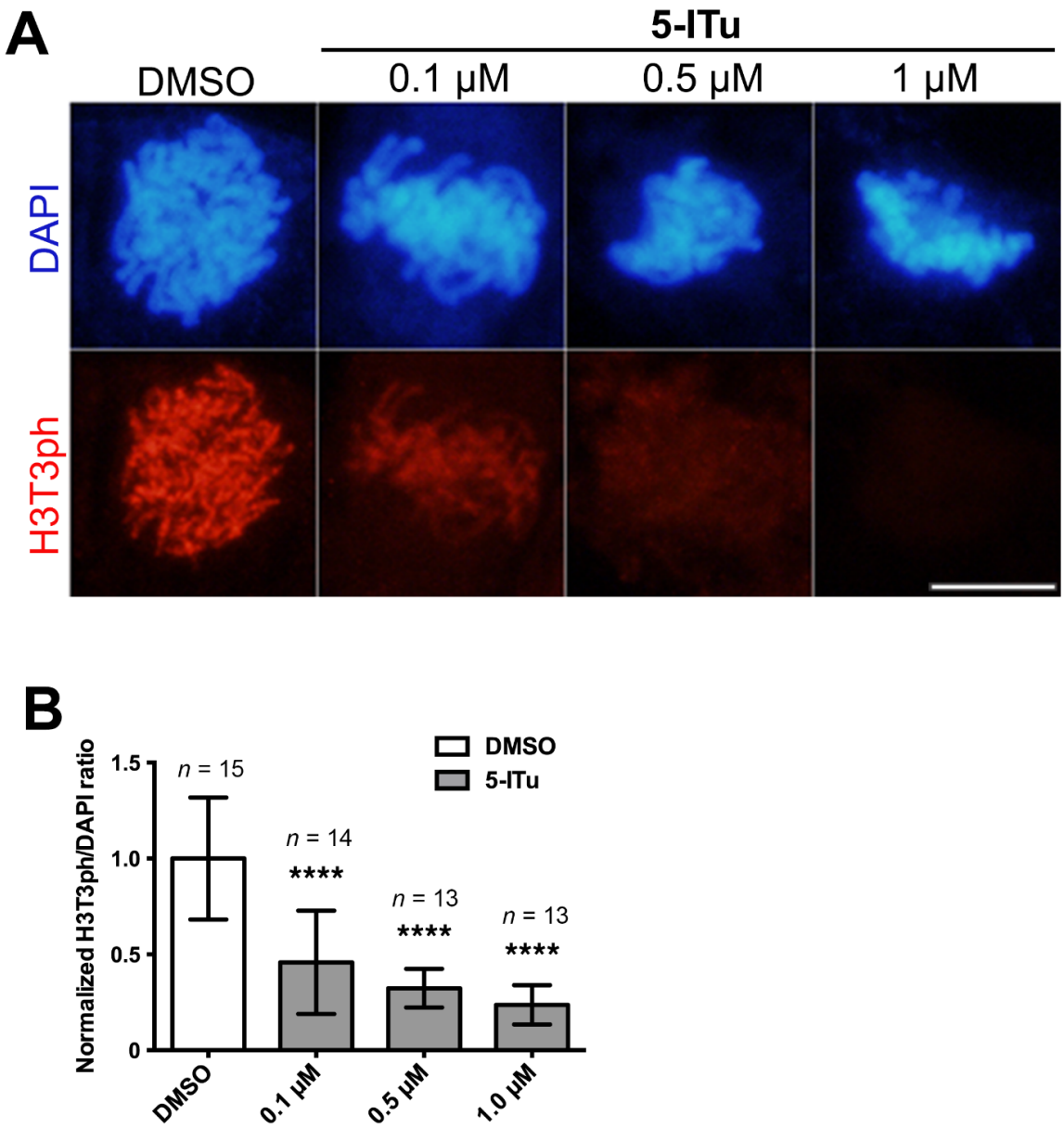

Figure 6. 5-ITu inhibits Haspin kinase function *in vivo*.

(A) Phosphorylation of histone H3 at Thr3 *in vivo*. After 24-hr incubation with indicated concentrations of 5-ITu, BY-2 cells were immunostained with anti-H3T3ph (red). DNA (blue) was stained with DAPI. Prometaphase stage is shown. Scale bar = 10 μm. (B) Quantitative data from experiment shown in B. Data was normalized to DAPI ratio. Values are mean ± SD. Asterisks indicate significant differences compared with control (DMSO) (****$p < 0.0001$; one-way ANOVA).

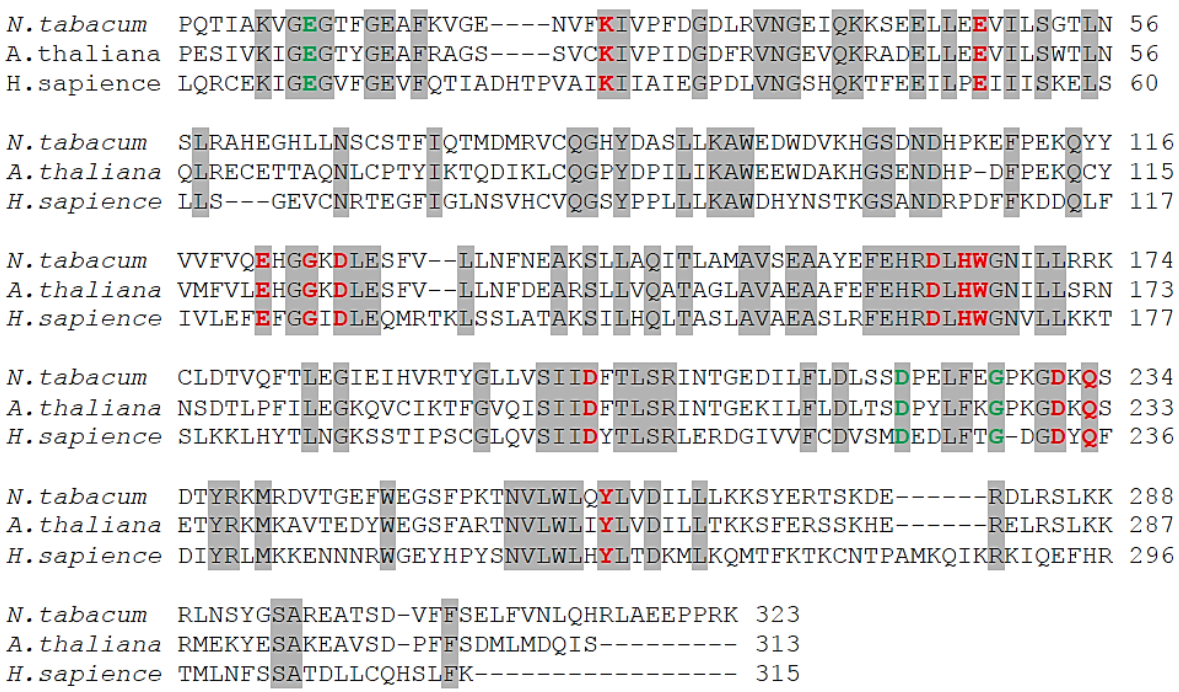

Figure 7. Multiple alignment of Haspin homologues in the kinase domain.

Alignment of the kinase domains of NtHaspin, AtHaspin and human Haspin. Conserved amino acids are shaded in gray, missing residues are shown in dashes. Residues binding ATP/Mg^{2+} are highlighted in red, important residues from the catalytic cleft for histone H3 phosphorylation are shown in green.

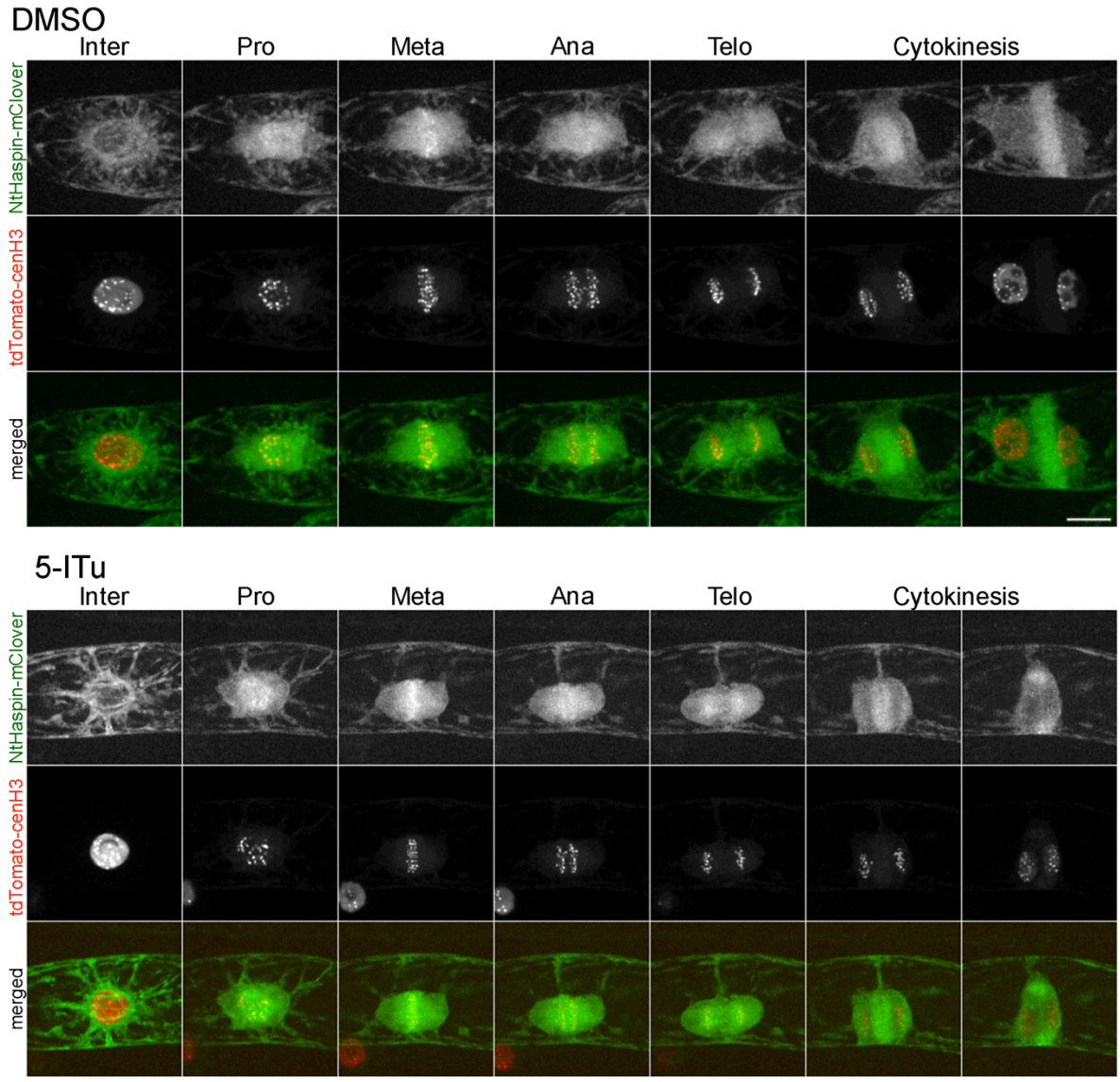

Figure 8. Subcellular distribution of NtHaspin-mClover through the cell cycle.

Localization of NtHaspin kinase in living BY-2 cells. Live-cell imaging was performed on BY-2 cells expressing NtHaspin-mClover and tdTomato-CenH3 (centromeres) after a 1-hr treatment with DMSO (control) or 1 μM 5-ITu. Merged images show NtHaspin (green) and centromeres (red). Images are maximum projections of z-stack. Scale bar = 10 μm.

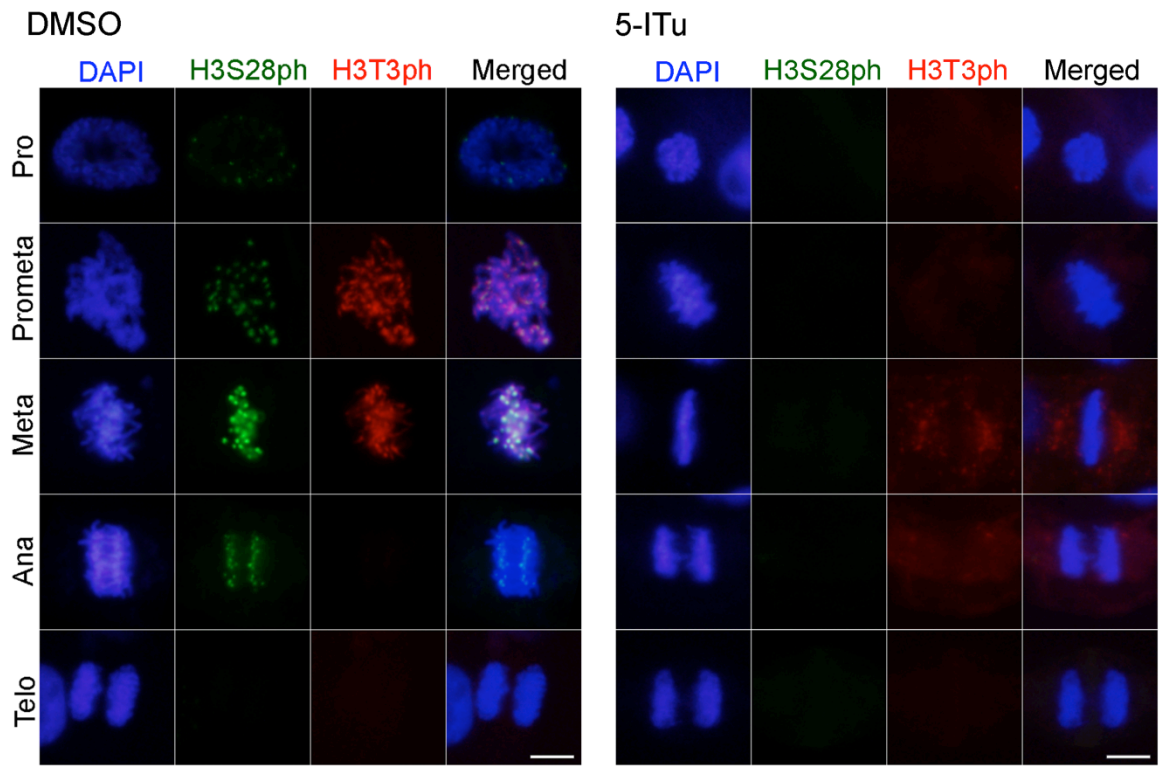

Figure 9. Phosphorylation of histone H3 at Thr3 throughout the cell cycle *in vivo*.

After 24-hr incubation with DMSO (control) or 1 μM 5-ITu, BY-2 cells were fixed and immunostained with the following antibody: H3T3ph (red), H3S28ph (green). DNA (blue) was stained with DAPI. Scale bar = 10 μm.

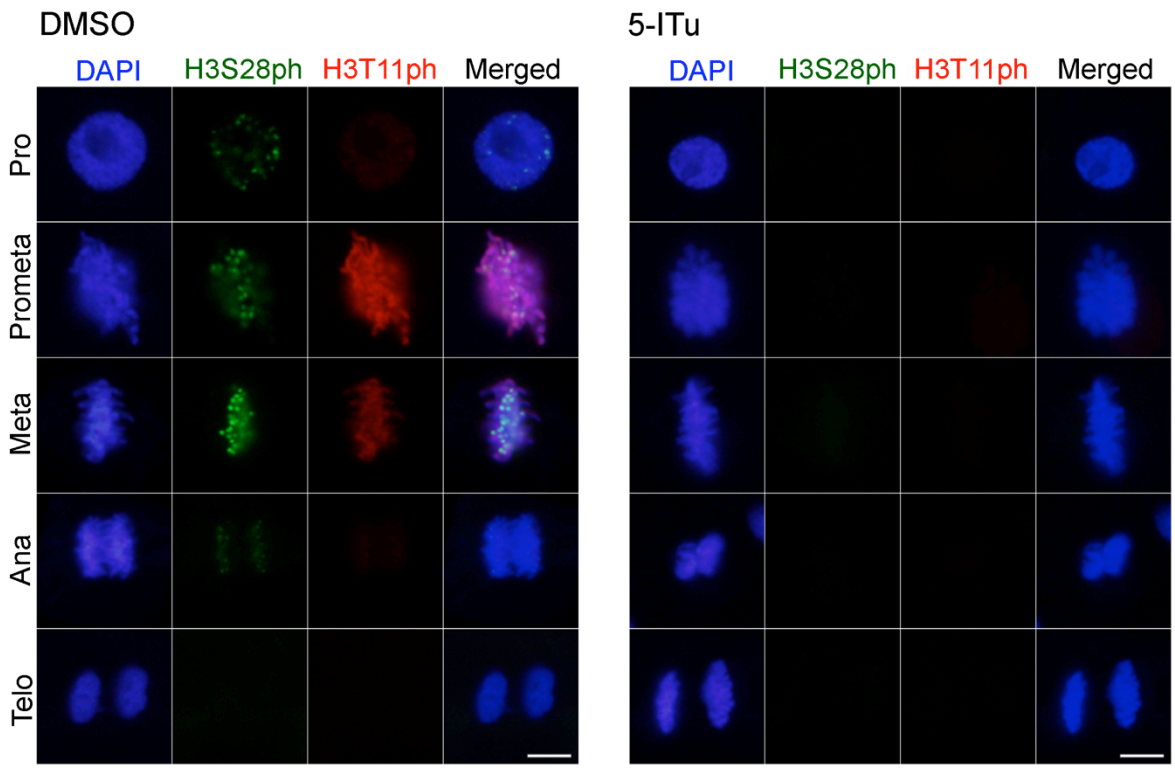

Figure 10. Phosphorylation of histone H3 at Thr11 throughout the cell cycle *in vivo*.

After 24-hr incubation with DMSO (control) or 1 μM 5-ITu, BY-2 cells were fixed and immunostained with the following antibody: H3T11ph (red), H3S28ph (green). DNA (blue) was stained with DAPI. Scale bar = 10 μm.

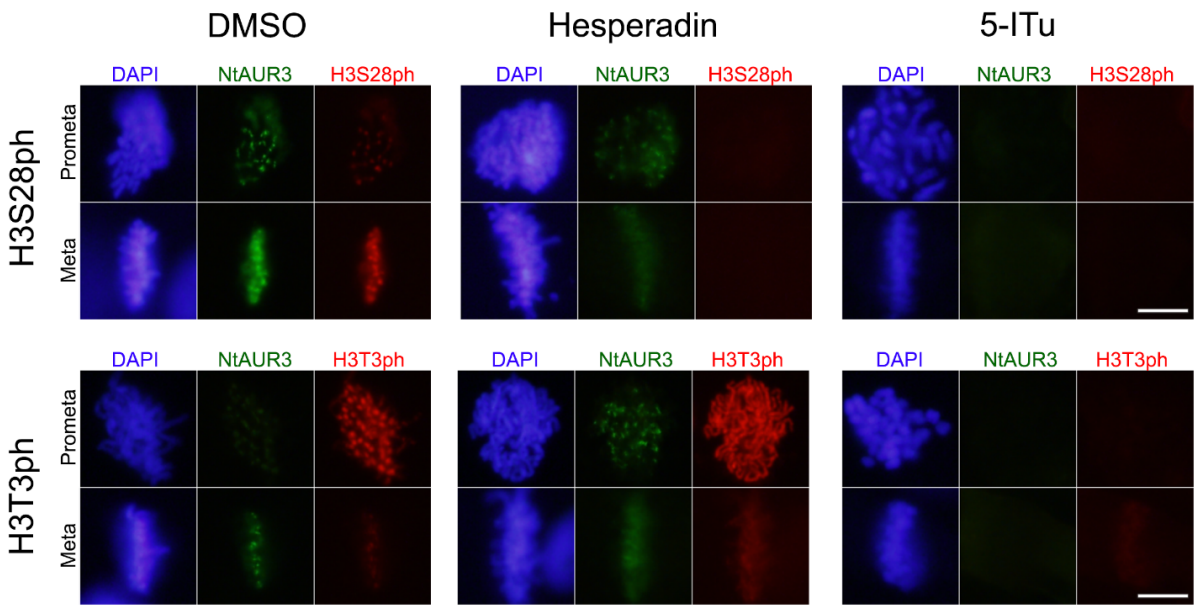

Figure 11. NtAUR3 accumulation on centromeres associated with NtHaspin function.

BY-2 cells expressing NtAUR3-mClover were incubated with DMSO (control), 1 μM 5-ITu, or 5 μM hesperadin. After 24 hr, cells were fixed and immunostained with antibody against H3T3ph (red) or H3S28ph (red). Anti-GFP antibody used to visualize NtAUR3-mClover (green), DNA (blue) was stained with DAPI. Prometaphase and metaphase are shown. Scale bar = 10 μm.

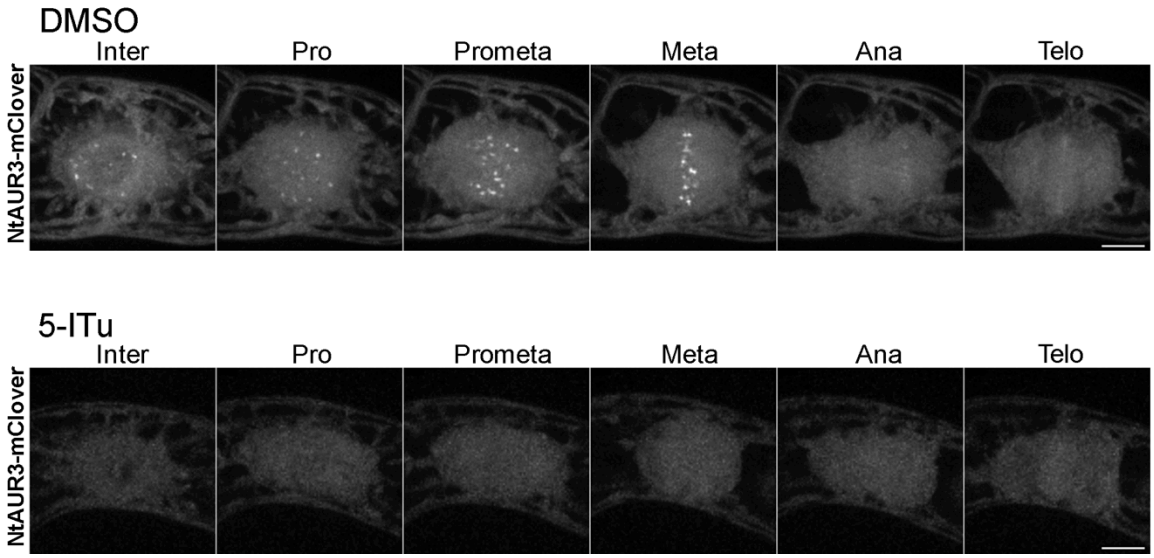

Figure 12. Distribution of NtAUR3 throughout cell cycle.

Live-cell imaging was performed on BY-2 cells expressing NtAUR3-mClover after a 1-hr treatment with DMSO (control) or 1 μM 5-ITu. Images are maximum projections of z-stack. Scale bar = 10 μm.

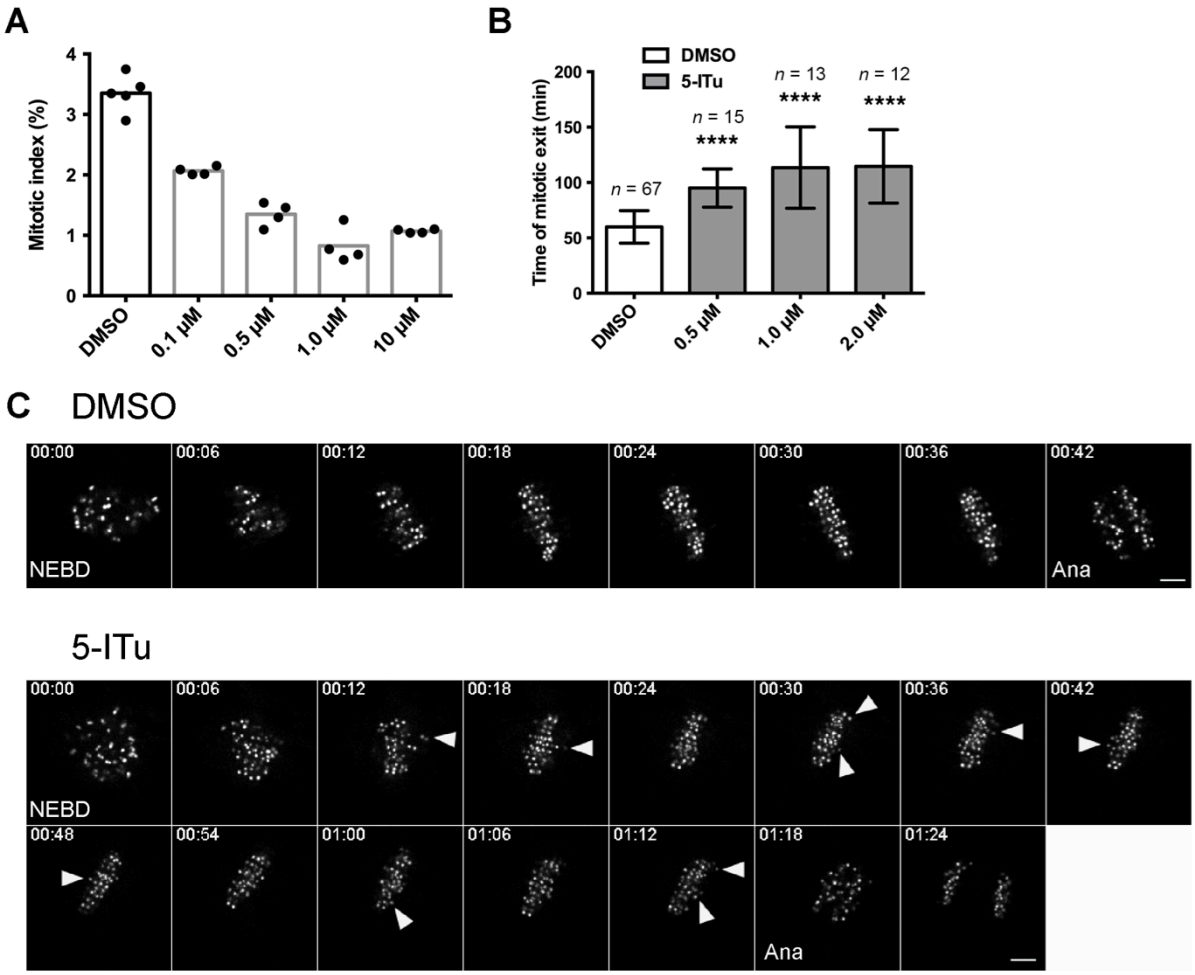

Figure 13. Effects of 5-ITu on BY-2 cell culture, mitosis duration, and chromosome alignment.

(A) BY-2 cells were treated with indicated concentrations of 5-ITu or DMSO (control) for 24 hr. Mitotic index was calculated from ≥ 1,000 cells in 4 independent experiments. Raw data is plotted on graph. (B) Average duration of mitosis (from pro- till telophase) was calculated from live-imaging data. Live-cell imaging was performed on BY-2 cells after a 1-hr treatment with DMSO (control) or indicated concentrations of 5-ITu. Graphs show mean value of ≥ 10 mitotic cells for each concentration ± SD. Asterisks indicate significant differences compared with control (DMSO) (**$p < 0.01$, ****$p < 0.0001$; one-way ANOVA). (C) Chromosome alignment in living BY-2 cells. Imaging was performed on BY-2 cells expressing tdTomato-CenH3 after a 1-hr

treatment with DMSO (control) or 1 μM 5-ITu. White arrowheads indicate lagging chromosomes under 5-ITu treatment. NEBD = nuclear envelope breakdown; Ana = anaphase. Images are maximum projections of z-stack acquired every 6 minutes. Scale bar = 10 μm

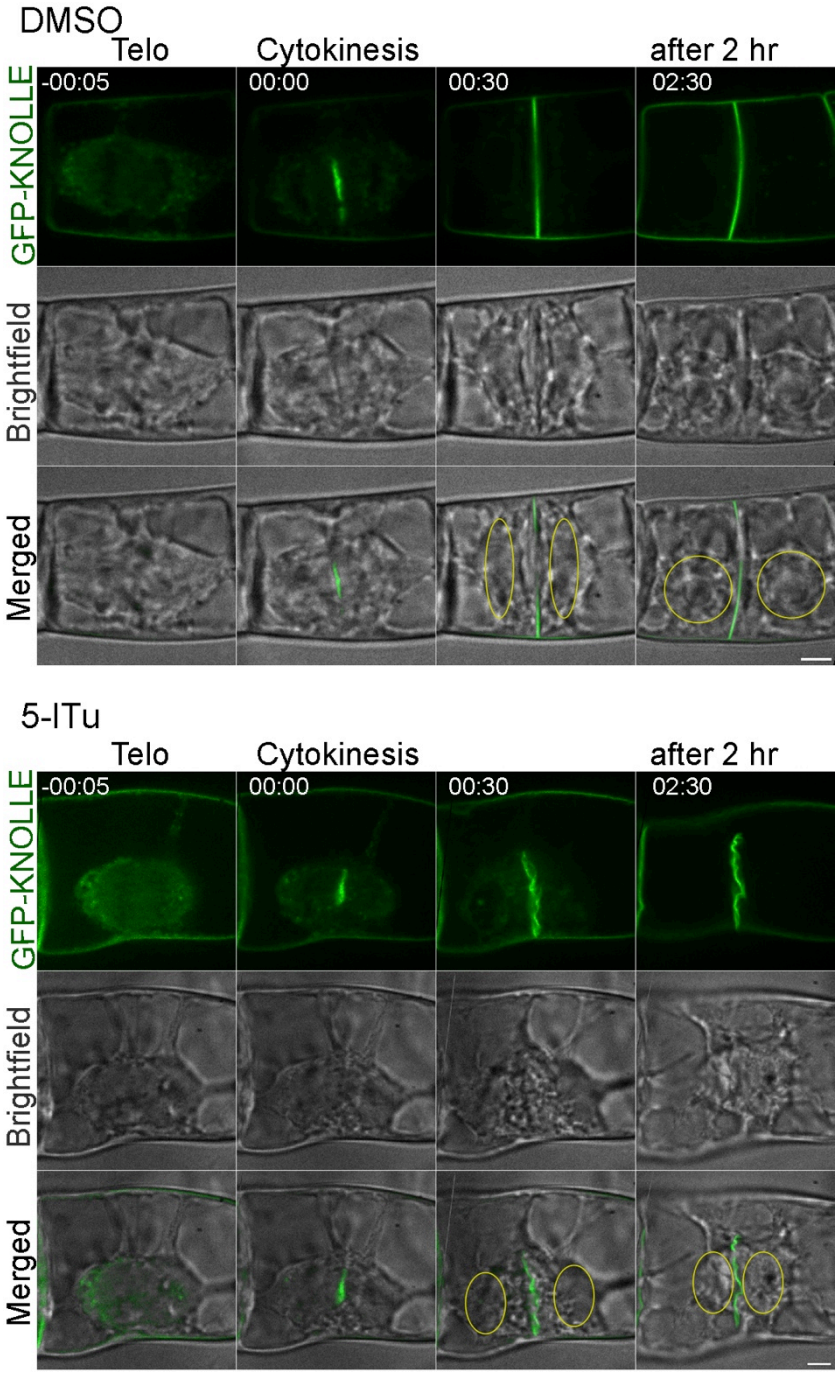

Figure 14. Cytokinesis defect in BY-2 cells under 5-ITu treatment.

Restriction of syntaxin KNOLLE to cell plate. Live-cell imaging was performed on BY-2 cells expressing GFP-KNOLLE after a 1-hr treatment with DMSO (control) or 1 μM 5-ITu. Incomplete cell plate formation can be observed under inhibitor treatment. Nuclei are labeled with yellow circles on merged plane. Images for GFP-KNOLLE and brightfield are a single focal plane. Numbers indicate time (hr: min); a starting point of cytokinesis, when GFP-KNOLLE was first observed on the cell plate, is shown at

00:00 column. Inter = interphase, Pro = prophase, Meta = metaphase, Telo = telophase. Scale bar = 10 μm.

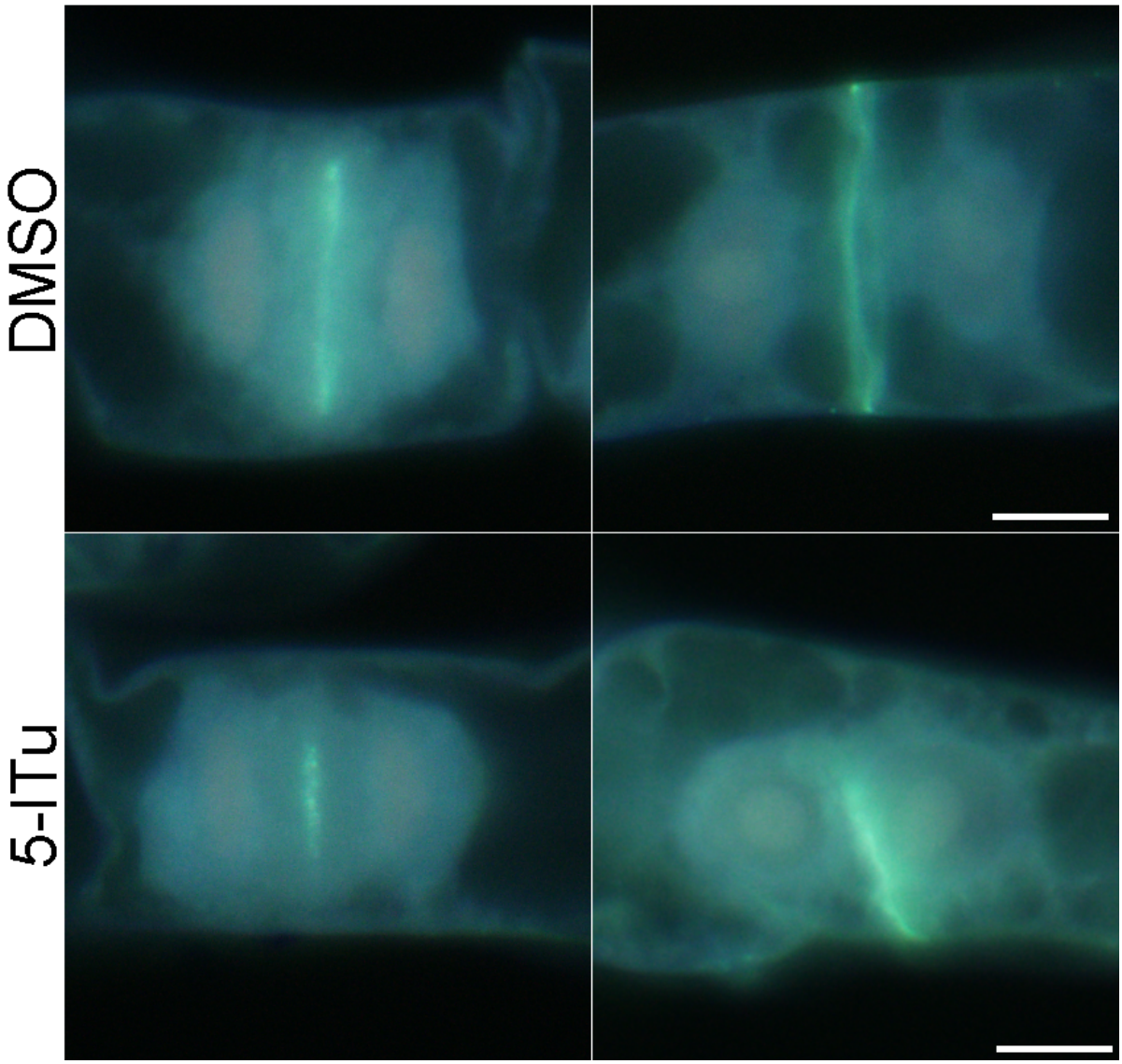

Figure 15. Accumulation of callose in the cell plate during cytokinesis.

BY-2 cell culture was incubated with 1μM 5-ITu or DMSO for 24 hr. Callose accumulation was detected by aniline blue staining. Scale bar 10 μm.

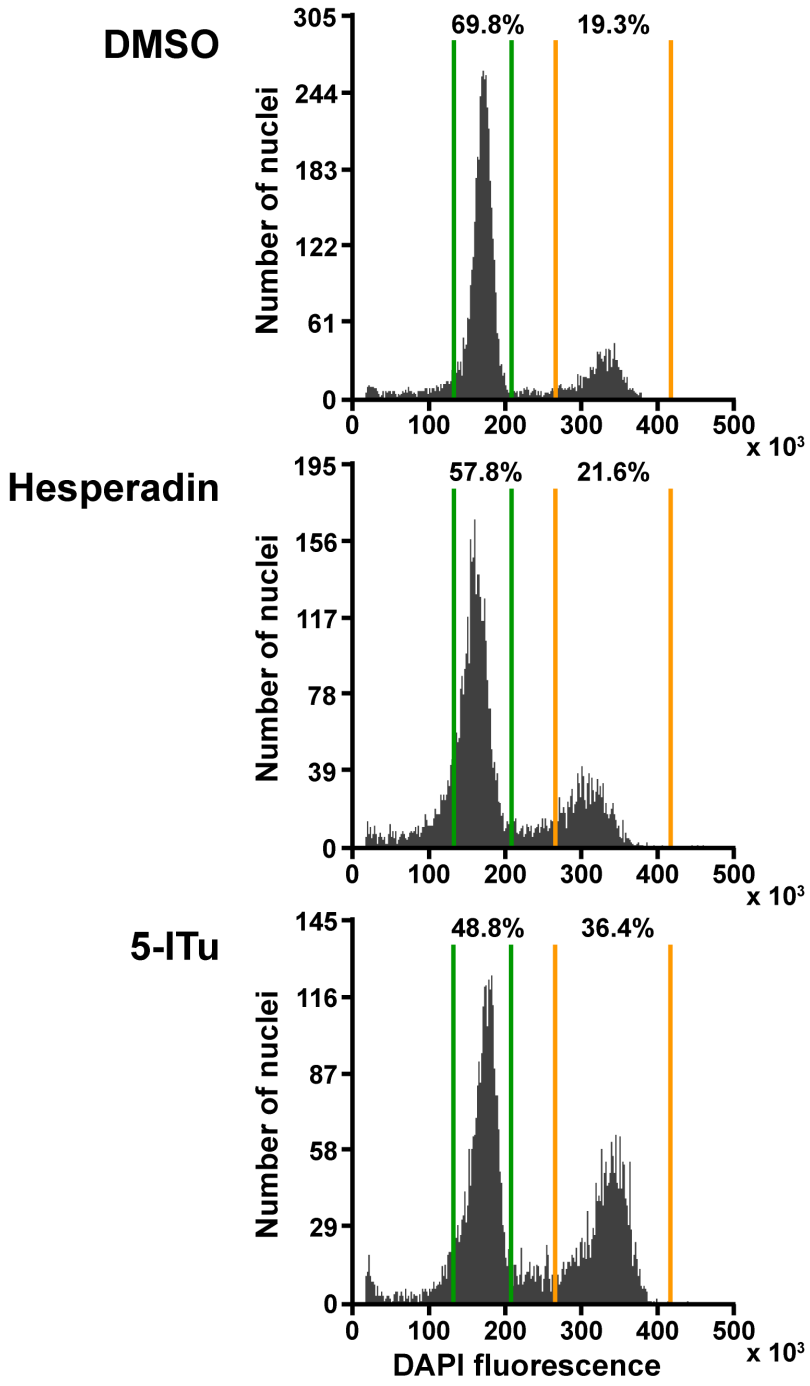

Figure 16. Analysis of cell culture ploidy after inhibitor treatment.

Cell treated for 24 hr with DMSO, 5 μM Hesperadin or 1 μM 5-ITu were analyzed by flow cytometry. First peak (in green lines) corresponds to 2C nuclei; second peak (in yellow lines) corresponds to 4C nuclei. Typical data from at least five independent measurements is shown.

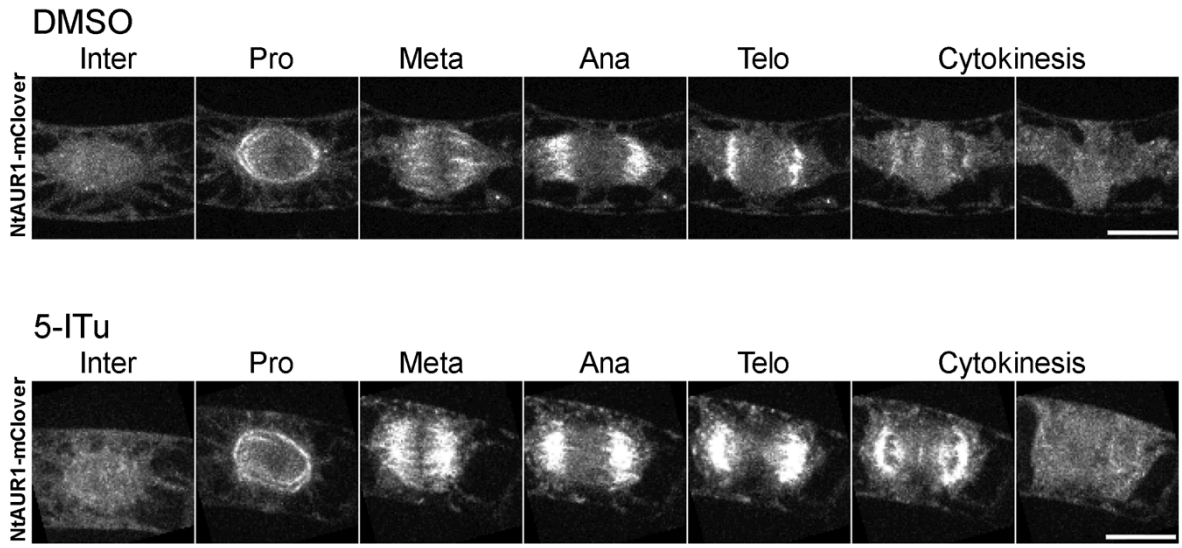

Figure 17. Distribution of NtAUR1 through the cell cycle.

Live-cell imaging was performed on BY-2 cells expressing NtAUR1-mClover after a 1-hr treatment with DMSO (control) or 1 μM 5-ITu. . Images are maximum projections of z-stack. Scale bar = 10 μm.

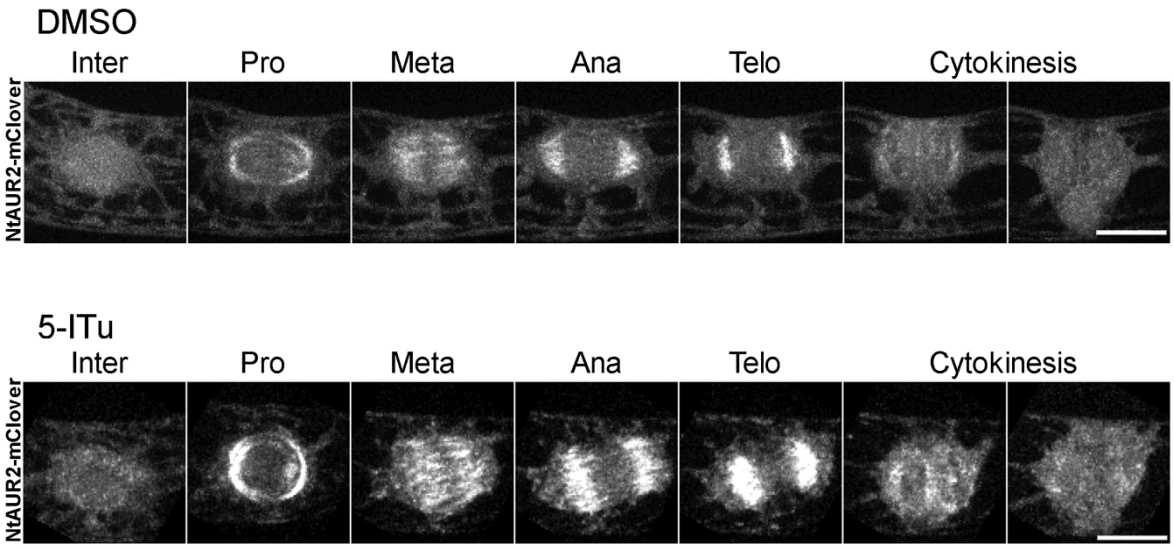

Figure 18. Distribution of NtAUR2 through the cell cycle.

Live-cell imaging was performed on BY-2 cells expressing NtAUR2-mClover (B) after a 1-hr treatment with DMSO (control) or 1 μM 5-ITu. Images are maximum projections of z-stack. Scale bar = 10 μm.

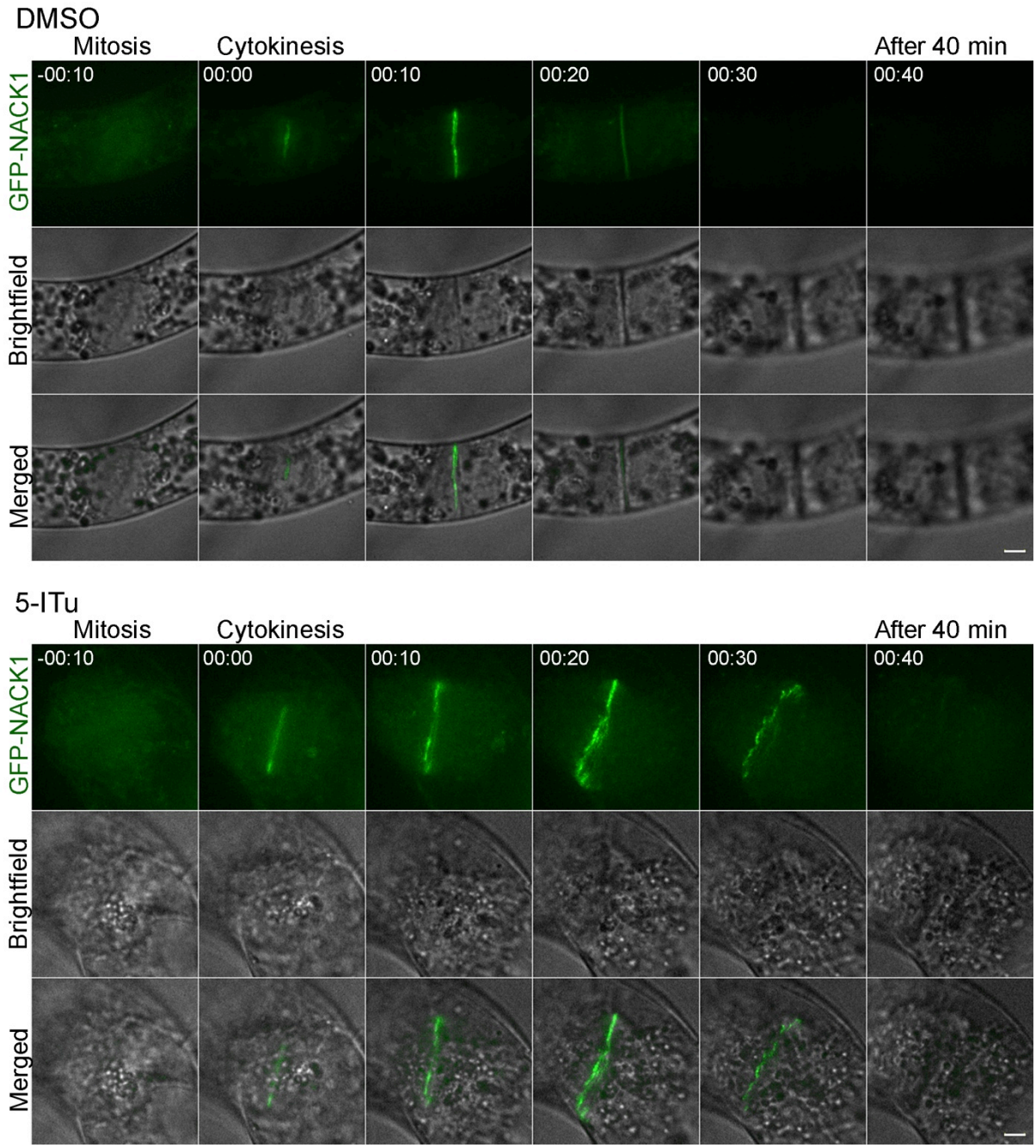

Figure 19. Distribution of kinesin NACK1 in BY-2 cells.

Distribution of NACK1 throughout the cell cycle. Live-cell imaging was performed on BY-2 cells expressing GFP-NACK1 after a 1-hr treatment with DMSO (control) or 1 μM 5-ITu. Images for GFP-NACK1 and brightfield are a single focal plane acquired every 10 min. Scale bar = 10 μm.

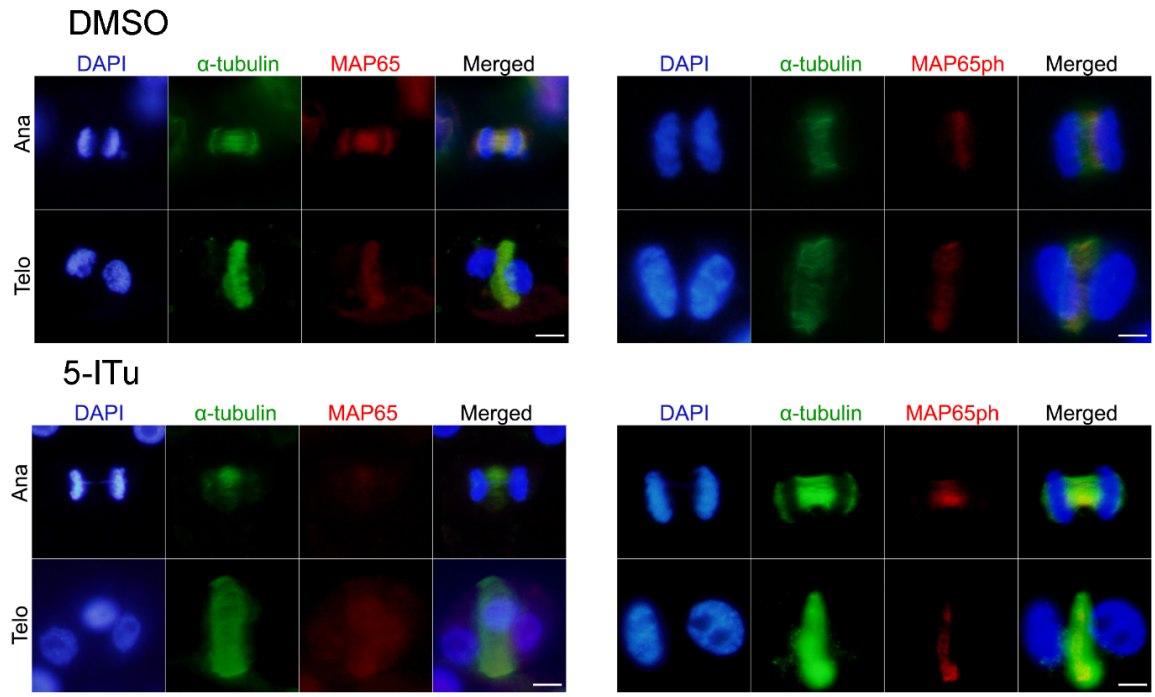

Figure 20. Localization and phosphorylation of downstream member from NACK-PQR pathway, MAP65-1.

Subcellular localization of NtMAP65-1 and NRK1-phosphorylated NtMAP65-1 during cytokinesis. BY-2 cells were treated with DMSO (control) or 1 μM 5-ITu 24 hr before fixation. Then, cells were stained with anti-MAP65-1 (red) or anti-MAP65-1ph (red) and anti-α-tubulin (green) antibodies. DNA (blue) was stained with DAPI. Scale bar = 10 μm.

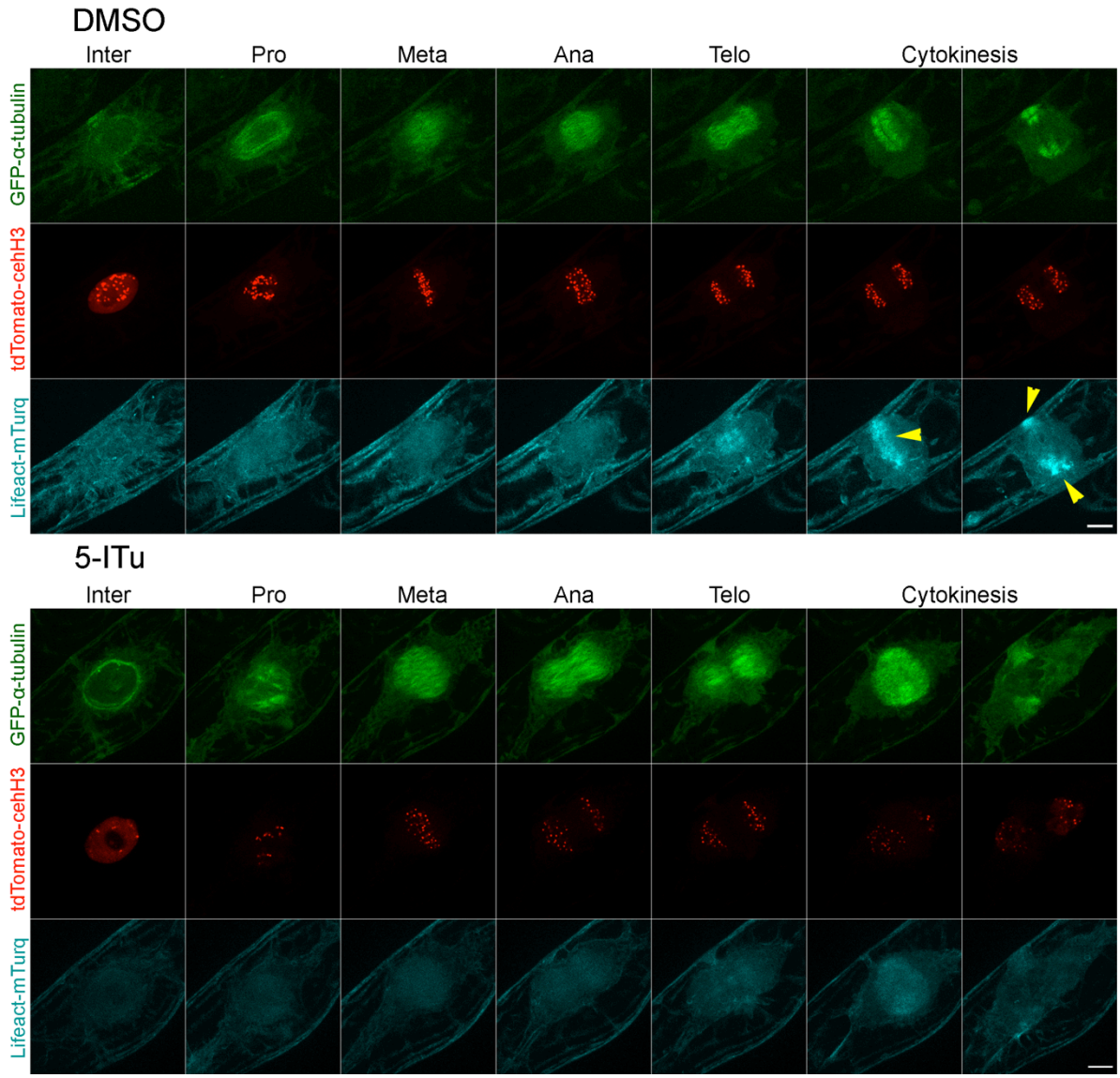

Figure 21. Distribution of microtubules and actin filaments during mitosis and cytokinesis.

Live-cell imaging was performed on BY-2 cells expressing GFP-α-tubulin (green), tdTomato-CenH3 (red), and Lifeact-mTurquoise2 (cyan) after a 1-hr treatment with DMSO (control) or 1 μM 5-ITu. Yellow arrows indicate concentration of actin at cell plate. Images are maximum projections of z-stack. Scale bar = 10 μm.

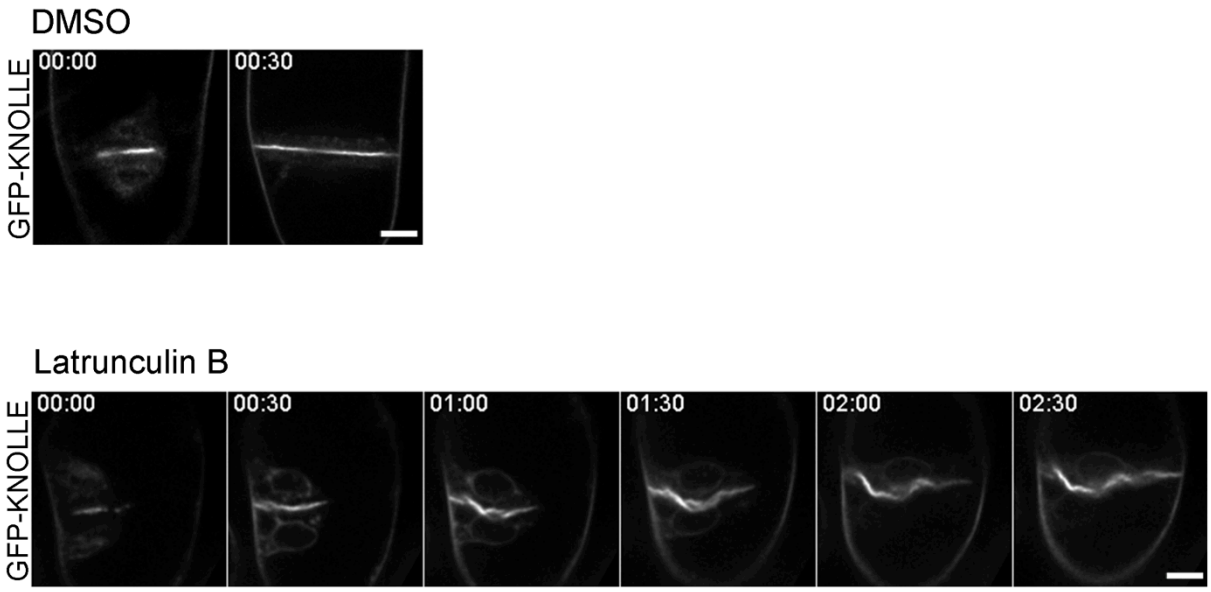

Figure 22. Cytokinesis in BY-2 cells with disrupted actin filaments.

Live-cell imaging was performed on BY-2 cells expressing GFP-KNOLLE after 3 hr treatment with DMSO (control) or 2 μM LatrunculinB. Images for GFP-KNOLLE are a single focal plane acquired every 30 min. Scale bar = 10 μm.

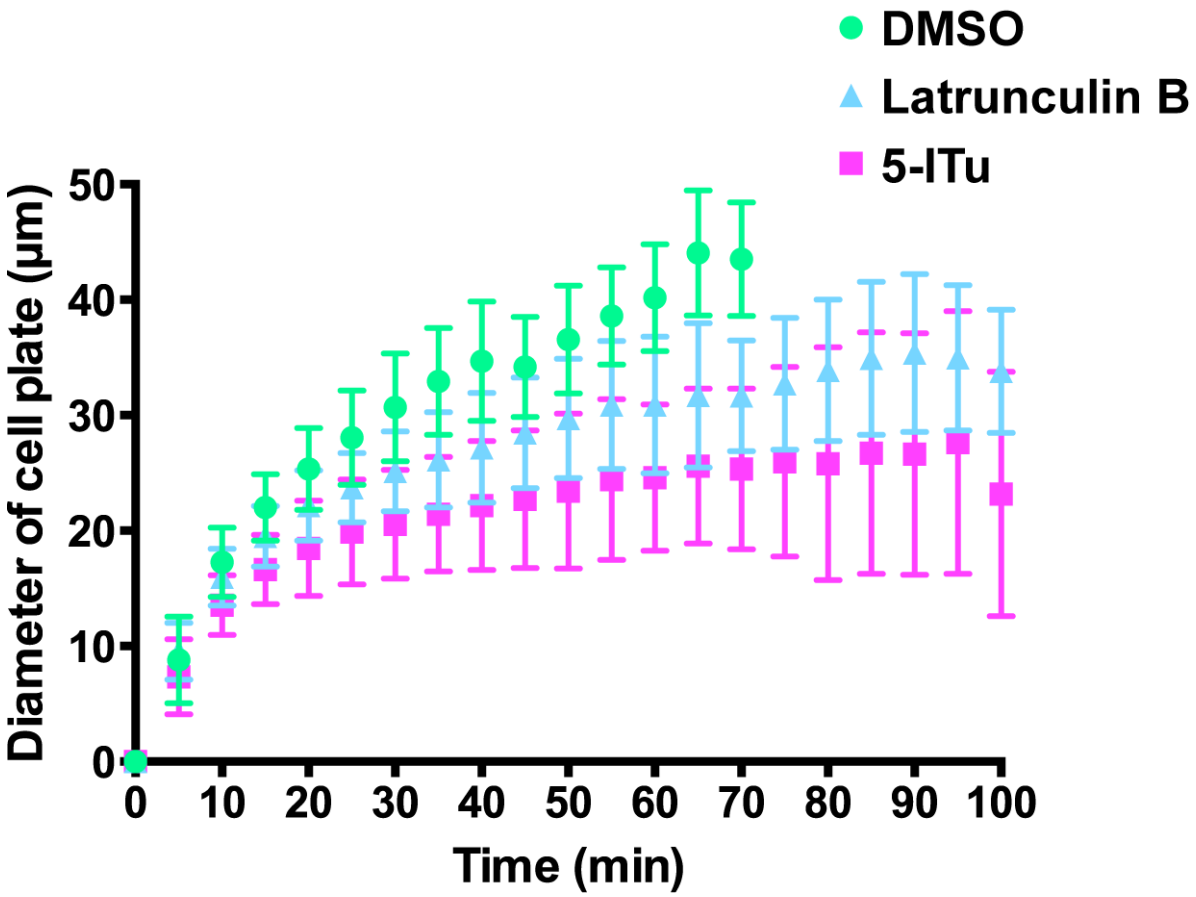

Figure 23. Cell plate expansion rate

Data was acquired from live-cell imaging in BY-2 cells expressing GFP-KNOLLE. Live-imaging was performed after 1 hr treatment with DMSO, 2 µM Latrunculin B or 1 µM 5-ITu and images were acquired every 5 min. Every time point shows mean value of the cell plate diameter ± SD, for ≥ 10 mitotic cells in each concentration.

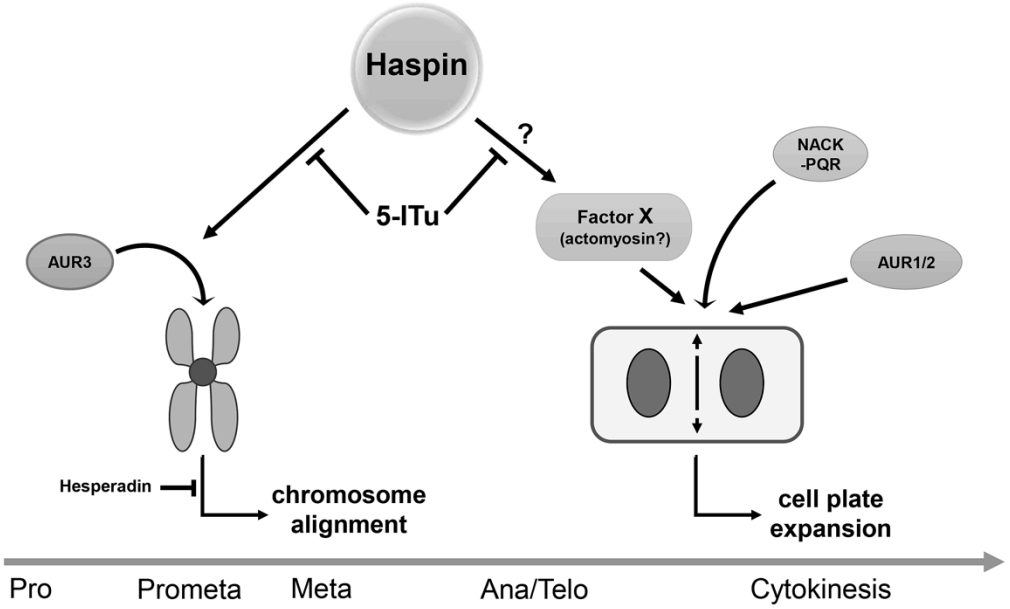

Figure 24. Schematic representation of the role of Haspin in regulating plant cell division.

Inhibition of Haspin function by 5-ITu disturbs centromeric localization of AUR3. AUR3 mislocalization leads to delay in chromosome alignment during prometa-/metaphase. Later, inhibition of Haspin leads to cytokinesis failure due to arrest of late cell plate expansion; however, known regulatory pathways including AUR1/2 and NACK-PQR cascade are unaffected. Hypothetical Factor X, potentially the actomyosin complex, is proposed as the intermediate link in the Haspin cascade.

Table 1

	Normal cytokinesis	Cytokinesis defect
DMSO	45	4
5-Itu	0	14

Table 1. Quantitative data for cytokinesis defect phenotype.

BY-2 cells expressing GFP-KNOLLE were treated with DMSO (control) or 1 μM 5-ITu. Live-imaging was for 24 hr and 8 view fields were selected and observed at each concentration. Cells with incomplete cell plate after 2 hr from the beginning of cell division were considered to have cytokinesis defect. Difference between DMSO and 5-ITu is significant ($p < 0.0001$ by two-tailed Fisher's exact test).

Table 2. The list of primers in this study

Name	Gene	Primer sequence
SalI-NtHaspin-F	NtHaspin	GTCGACATGACTTCTCGAGCGGCTGATTTGTGGGCAG
BglII-NtHaspin-R		AGATCTTTTTCTTGGGGGTTCTTCTGCCAGTCGGTGC
SalI-NtAUR1-F	NtAurora1, NtAurora2	GTCGACATGGGGATCGCAACGGAGAATCAACCACAGC
BglII-NtAUR1/2-R		AGATCTGCCCTTATAAACACCTGAAGGATCTGCATTC
SalI-NtAUR2-F	NtAurora2	GTCGACATGGCGATTGCAGCGGAGACTCAGCCACAG
SalI-NtAUR3-F	NtAurora3	GTCGACATGGCTTCCAAAACTCAAATTCATCCTCC
BamHI-NtAUR3-R		GGATCCACCAGTGACATTCTTAATTATCCAAGG
SalI-Lifeact-F	Lifeact	GTCGACATGGGCGTGGCCGACCTGATCAAGAAG
NheI-mTurquoise-R		GCTAGCTTACTTGTACAGCTCGTCCATGCCATG

ABBREVIATIONS

Abbreviation	Definition
5-ITu	5-Iodotubercidin
Ana	Anaphase
ANOVA	analysis of variance
AUR1	Aurora1 kinase
AUR2	Aurora2 kinase
AUR3	Aurora3 kinase
BY-2	Bright Yellow-2
CenH3	centromeric histone H3
CPC	chromosome passenger complex
DMSO	dimethyl sulfoxide
GFP	green fluorescent protein
GST	glutathione S-transferase
H3S10ph	phosphorylation of histone H3 at serine10
H3S28ph	phosphorylation of histone H3 at serine28
H3T11ph	phosphorylation of histone H3 at threonine11
H3T3ph	phosphorylation of histone H3 at threonine3
Hes	Hesperadin
Inter	Interphase
LatB	Latrunculin b
MAP65	microtubule-associated protein 65

Meta	Metaphase
MT	mictrotubules
NEBD	nuclear envelope breakdown
Pro	Prophase
Prometa	Prometaphase
tdTomato	tandem dimer Tomato
Telo	Telophase

CONCLUSION AND PERSPECTIVE

This thesis investigates a role of the mitotic kinase Haspin in plant regulatory network. While regulation of the cell division is a highly active area of animal research, many aspects of this process in plants remain a mystery. Haspin kinase homologues have been found in different living organisms, from nematodes to mammals, from fungi to plants. Proteins conserved through evolution are often required for basic cellular functions, thus to understand their role and interactions means to understand molecular machinery that maintains life. Significance of research dedicated to how, when and where such proteins operate, needs no explanation. In this study I aimed to make my contribution to the current knowledge of regulating cell division in plants.

My research was conducted mostly on tobacco BY-2 cell culture, recognized as a conventional model object for plant cell division studies. I inhibited the function of NtHaspin, using ATP-competitive inhibitor 5-ITu, and analyzed the effects by various methods, including cell live-imaging, *in vitro* and *in vivo* assays, FACS, etc. The obtained data can be roughly grouped into two parts, each corresponding to different stages of cell division and NtHaspin role during those stages.

I observed that inhibition of NtHaspin function increased length of mitosis due to delay in chromosome alignment in prometa/metaphase stages. Furthermore, localization and function on the centromeres of another mitotic kinase, NtAUR3, was disrupted in conformity with NtHaspin inhibition. Taking into consideration

literature data, I suggest that NtAUR3 and NtHaspin kinase are involved into the same regulatory pathway, responsible for chromosome alignment, wherein NtHaspin is located upstream and is essential for NtAUR3 proper functioning.

In addition, I propose that NtHaspin is related to cytokinesis process, since inhibition of NtHaspin function frequently resulted in serious defects of the cell plate expansion. Apparently, well-known factors involved into cytokinesis in plants, including microtubules, NACK-PQR pathway and AUR1/2, were not affected by NtHaspin inhibition. Therefore NtHaspin target and interactions in cytokinesis remain unclear and call for more research, although actomyosin seems to be a prospective candidate for further investigation.

This study presents strong evidence that Haspin kinase is an important factor in plant regulatory network. However, there are several more questions that should be addressed in future studies. Chemical inhibitor promiscuity presents a significant challenge and complicates result interpretation. However, in case of Haspin kinase, knockout is very likely to result in lethal phenotype. I suggest down-regulating Haspin function by siRNA in order to check 5-ITu-induced phenotype. Here, I demonstrated the relation between Haspin and AUR3 kinases, but how Haspin recruits AUR3 on centromeres is not clear yet. Another exciting and promising topic appears to be Haspin role in cytokinesis, which can be novel discovery for plant cell biology.

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