UNIVERSITÀ DEGLI STUDI DI MILANO

Scuola di Dottorato in Scienze Biologiche e Molecolari

XXV Ciclo

# A NEW HASPIN ROLE AT THE INTERSECTION BETWEEN MITOSIS AND CELLULAR POLARIZATION

**D PANIGADA**

PhD Thesis

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Academic year: 2012-2013

SSD: [BIO/11]

Thesis performed at Dipartimento di Bioscienze

# Contents

# Part I

# Abstract

Haspin is an atypical protein kinase; in several organisms it phosphorylates histone H3 on Thr3 and is involved in chromosome segregation. In *Saccharomyces cerevisiae,* H3Thr3 phosphorylation has never been observed and the function of haspin is unknown. We report that deletion of both *ALK1* and *ALK2,* encoding the haspin paralogs, causes the mislocalization of polarisome components. Following a transient mitotic arrest, this leads to an overly polarized actin distribution within the bud, where the mitotic spindle is consequently pulled. Here, spindle elongates generating anucleated mothers and binucleated daughters. Reducing the intensity of the bud-directed pulling forces partially restores proper cell division, suggesting that haspin controls the localization of polarity cues to preserve the coordination between polarization and the cell cycle, and to tolerate transient mitotic arrests. The evolutionary conservation of haspin and of the polarization pathways suggest that this function of haspin may be likely shared with other eukaryotic cells. We thus investigated a possible conservation of this control mechanism in mammalian cells, where indeed we found that haspin governs the orientation of the mitotic spindle thanks to its contribution to actin organization.

# State of the Art

**Overview of the Cell Cycle**

The cell cycle comprises the series of events that lead a cell to its duplication and subsequent division. It consists of four distinct phases: G1 phase, S phase, G2 phase and M phase; the first three ones are collectively known as interphase and may last for variable periods of time depending on the cell type.

To provide a complete and correct genetic information to the daughter cell, the mother has to duplicate and segregate accurately its genome. The first process is performed during the S phase (Synthesis), when DNA is replicated and is retained into the nucleus until M phase (Mitosis), when the two copies of the genome are segregated. Synthesis and Mitosis are separated by two Gap phases (G1 between M and S and G2 between S and M), during which the cell prepares for the following step of the cell cycle.

M phase is subdivided in different steps: prophase, during which DNA is condensed, the nuclear membrane is dissolved and the mitotic spindle is assembled; metaphase, when chromosome are aligned in the middle of the

cell; anaphase, when the chromosome segregation takes place; telophase, during which chromosomes are decondensed and the mitotic spindle is disassembled and finally cytokinesis, when the cell divides in half generating two distinct daughter cells.

During G1, if the environmental conditions are not appropriate for growth and division, cells can reversibly exit from the cell cycle and enter a resting phase (G0),. When better conditions are re-established, cells in G0 can re-enter the cell cycle and proceed in S phase.

### *Saccharomyces cerevisiae* Cell Cycle

Budding yeast (*Saccharomyces cerevisiae*) is a non pathogenic unicellular fungus belonging to the ascomycetes family. It has a short duplication time and a small genome (12 Mbp) which is divided in 16 chromosomes.

Although small, this organism maintains much of the functional and structural complexity of higher eukaryotes. This aspect, combined with its genetic versatility, makes it an invaluable model organism to understand the molecular details of complex biochemical mechanisms, such as the control of the cell division, which occurs by budding. Yeast cells display morphological characters typical of the cell cycle phase in which they are.

Initial studies on its cell cycle regulation determined that initiation of the cell cycle is controlled in late G1 and at a precise moment called START (Hartwell et al., 1970). After passing START, cells cannot turn back and have to complete the entire cell cycle. After G1, DNA is replicated during S phase, followed by G2 and, finally, M phase that leads to chromosomes segregation into daughter cells.

In G1, *S. cerevisiae* exists morphologically as a single unbudded cell. As soon as it goes through the START, a small bud emerges and grows in a polarized manner. While during S phase the bud grows apically, in G2 its growth shifts from polarized to isotropic, until it reaches a size approximately equal to its mother.

Cell cycle progression is regulated by key players, the CDKs (Cyclin Dependent Kinases), whose activity requires the formation of a complex with regulatory factors, called cyclins, (Pines, 1995). Six CDKs are present in *S. cerevisiae*: Cdc28 (also known as Cdk1), Pho85, Kin28, Ssn3, Bur1 and Ctk1 (Simon et al., 1986; Liu and Kipreos, 2000; Yao et al., 2000; Huang et al., 2007). *CDC28* was initially identified in a screening for genes that control the cell cycle (Hartwell et al., 1973). It is the most important CDK, and has an essential role in cell cycle progression, even though many of its functions, especially in the earlier phases of the cell cycle, are supported by the non-essential CDK Pho85 (Huang et al., 2007). The other CDKs are thought to function mainly in the process of transcription (Meinhart et al., 2005).

*CDC28* encodes for a 34 kDa serine-threonine kinase, whose activity is tightly regulated by the interaction of two classes of different cyclins (Pines, 1995; Wittenberg, 2005): the G1 cyclins, Cln1-3; and the B-type cyclins, Clb1-6 (Fig. 1) (Futcher, 1996; Lew and Kornbluth, 1996; Nasmyth, 1996). Interaction of these cyclins with Cdc28 is essential for obtaining a functionally active kinase and to find and select its substrates (Peeper et al., 1993).

During G1, Cdc28 is inactive, due to low cyclin concentrations and to the presence of the CDK inhibitors (CKIs) Sic1 and Far1 (Schwob and Nasmyth, 1993). In late G1, when a critical cell size has been reached, the level of Cln cyclins rise leading to the increase of CDK activity and allowing passage

through START (Peter and Herskowitz, 1994; Verma et al., 1997). Transcription is crucial for this step; Cdc28-Cln3 activates transcriptional programs that stimulate the expression of nearly 200 genes (collectively referred to a the G1 cluster), among which are *CLN1* and *CLN2* (Marini and Reed, 1992; Tyers et al., 1993; Dirick et al., 1995; Stuart and Wittenberg, 1995) (Spellman et al., 1998; Cho et al., 2001).

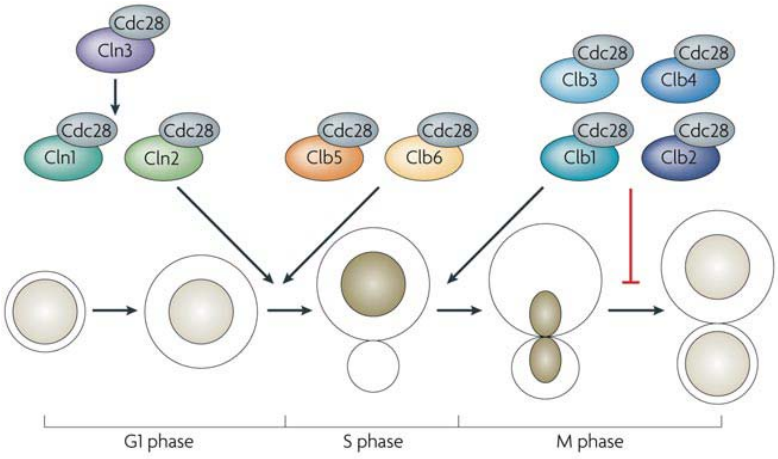


Fig. 1. *S. cerevisiae* cyclins and CDK (Bloom and Cross, 2007)

Two transcriptional factors are activated by Cdc28 in order to modulate the expression of the G1 cluster: MBF (Mlu1-box binding factor), which binds promoters with the MCB (Mlu1 cell cycle box) promoter element and SBF, which binds promoters with the SCB element (Swi4/6 cell cycle box). The first one induces the transcription of the genes involved in DNA replication and repair, while SBF regulates the transcription of the genes involved in cell cycle progression, cell morphogenesis and spindle pole body duplication (Wittenberg and Reed, 2005). During early G1, promoter-bound SBF is kept inactive by Whi5 (Costanzo et al., 2004; de Bruin et al., 2004). Initial

phosphorylation of Whi5 by Cdc28 and Pho85 leads to its dissociation from SCB promoters, allowing SBF to induce expression of its target genes (Huang et al., 2009; Takahata et al., 2009), among which there are *CLN1* and *CLN2*. The formation of a complex between Cln1, Cln2 and Cdc28 triggers the actin polarization pathway and the duplication of the spindle pole body (SPB); moreover, it promotes the degradation of the Sic1 inhibitor (Lew and Reed, 1993, 1995; Schneider et al., 1996; Haase et al., 2001), preparing the cell to undergo S phase.

G1 cyclins promote also the expression of the first group of B-type cyclins, Clb5 and Clb6, that are involved in initiation and control of DNA replication (Schwob and Nasmyth, 1993; Schwob et al., 1994; Geymonat and Spanos, 2004).

During S phase, Clb3 and Clb4 are expressed to prepare for the assembly of the mitotic spindle (Fitch et al., 1992). Lastly, at the beginning of the M phase Clb1 and Clb2 are expressed until the end of mitosis. These cyclins are involved in the mitotic progression, spindle elongation, nuclear division and segregation, and in the switch from polarized to isotropic growth (Fitch et al., 1992; Lew and Reed, 1993). Mitotic exit and the entry into a new G1 phase are triggered by degradation of B-type cyclins, and by de-phosphorylation of specific CDK substrates by Cdc14 phosphatase (see below for details).

**Mitosis**

Prior to mitosis, the Cdc28-Clb complex is kept inactive by an inhibitory phosphorylation on the well conserved residue Tyrosine 19 performed by Swe1 in *S. cerevisiae* (Sia et al., 1998). After S phase, Swe1 is exported from the nucleus and is recruited to the bud neck, where it is hyperphosphorylated;

this modification is a prerequisite for Swe1 ubiquitin-mediated degradation, and is requested for efficient Cdc28-Clb activation (Sia et al., 1996, 1998).

Once activated, Cdc28 prepares its inactivation by phosphorylating the Anaphase Promoting Complex / Cyclosome (APC/C). This modification enables the interaction of APC/C with its cofactor Cdc20, allowing the formation of an E3 ubiquitin-ligase complex that promotes the degradation of many mitotic targets via proteasome (Irniger et al., 1995; King et al., 1995; Sudakin et al., 1995; Peters, 2002). Among these targets there are B-type cyclins, whose degradation inactivates Cdc28, thus stimulating the exit from mitosis (King et al., 1995; Sudakin et al., 1995). The other major target of $APC/C^{Cdc20}$ is the securin Pds1, which normally inhibits the separase Esp1 (Cohen-Fix and Peters, 1996). Removal of securin allows Esp1 to cleave the cohesin complex subunit Scc1, promoting sister chromatids separation and nuclear division (Ciosk et al., 1998; Uhlmann et al., 1999, 2000).

The other biochemical event that contributes to end mitosis is the dephosphorylation of CDK substrates, promoted by the Cdc14 phosphatase (Visintin et al., 1998). This protein is usually kept inactive through its binding to an inhibitor, Cfi1 (also known as Net1), that, during interphase, retains the phosphatase into the nucleolus (Traverso et al., 2001).

Two regulatory networks act sequentially to promote the release and activation of Cdc14 (Fig. 2): the first is called FEAR (cdc *f*ourteen *e*arly *a*naphase *r*elease) and the second is the MEN (*m*itotic *e*xit *n*etwork) (Stegmeier and Amon, 2004; Rock and Amon, 2009).

The FEAR pathway acts in early anaphase, when CDK activity is still high, and depends upon Pds1 degradation by the APC (Stegmeier et al., 2002; Sullivan and Uhlmann, 2003); indeed, beyond its proteolytic role, Esp1 seems to inhibit

directly the PP2A phosphatase allowing Cfi1 phosphorylation by Cdc28 and the Cdc5 polo-like kinase (Shou and Azzam, 2002; Yoshida and Toh-e, 2002; Sullivan and Uhlmann, 2003; Azzam et al., 2004; Queralt et al., 2006). Modification of Cfi1 is responsible for the initial release of Cdc14 from the nucleolus into the nucleoplasm, where it promotes the elongation of the mitotic spindle by dephosphorylating the spindle midzone proteins Ase1 and Fin1 (Higuchi and Uhlmann, 2005; Woodbury and Morgan, 2006; Khmelinskii and Lawrence, 2007). Esp1 is not the only important factor of the FEAR network, indeed genetic analysis have implicated also *SPO12, SLK19,* as well as the polo-like kinase Cdc5 in this pathway (Stegmeier et al., 2002).

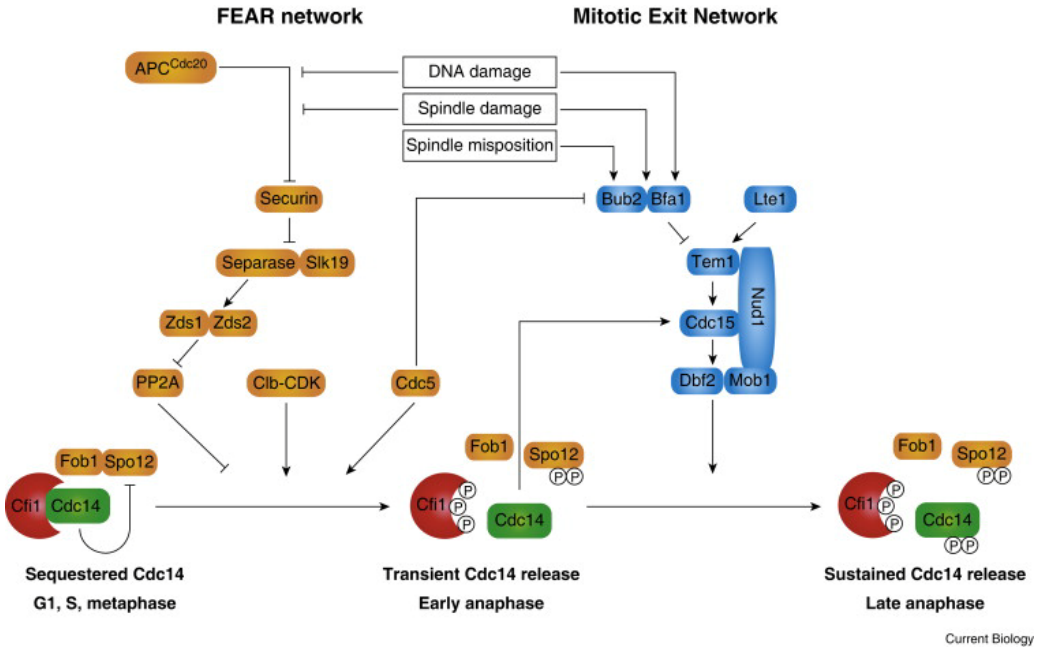


Fig. 2. *S. cerevisiae* pathways for Cdc14 release (Rock and Amon, 2009)

The decrease in Cdc28 activity during anaphase progression makes it difficult to sustain high Cfi1 phosphorylation levels, and in the absence of other events,

Cdc14 is resequestered into the nucleolus. Full activation of Cdc14 therefore requires activation of the MEN essential pathway. The apical activator of this network is the GTPase Tem1 that, once activated, triggers a kinases cascade which induces the dissociation of Cdc14 from Cfi1, thereby allowing the complete release of Cdc14 in the cytoplasm (Shirayama et al., 1994; Toyn and Johnston, 1994; Mah et al., 2001). Once in the cytoplasm Cdc14 is free to dephosphorylate all its targets and finally promote mitotic exit (Queralt and Uhlmann, 2008). Tem1 is negatively regulated by a GAP (GTPase Activating Protein) complex, composed by Bub2-Bfa1, while a protein with a GEF (Guanine nucleotide Exchange Factor) function for the GTPase has not been discovered yet (Geymonat et al., 2002, 2009). However, it is clear that activation and inhibition of Tem1 are finely regulated by the spindle position checkpoint (SPOC), which will be discussed later.

**Spindle checkpoints**

Every step of the cell cycle must proceed in a precise order and only after the previous events have reached completion. To this aim, eukaryotic cells have developed highly conserved mechanisms called “checkpoints” that monitor the completion of critical cell cycle events and the integrity of specific cellular structures, and modulate cell cycle progression.

In *S. cerevisiae* two checkpoints act during mitosis, ensuring the correct partiotining of the genomic material: the SAC (Spindle Assembly Checkpoint) and the SPOC (Spindle Position Checkpoint).

The SAC is an evolutionary conserved checkpoint active in prometaphase that prevents the precocious separation of sister chromatids (Musacchio and

Salmon, 2007). More specifically, the SAC monitors that the kinetochores of the two sister chromatids are properly attached to the opposite poles through microtubules, in a configuration that is called bi-orientation. This allows the application of equal pulling forces acting on the kinetochores that become under tension (Fig 3) (Dewar et al., 2004). The SAC coordinates the microtubule-kinetochore tension with anaphase entry, by negatively regulating the Cdc20 activator of APC/C. Through the inhibition of the Cdc20 activity, the SAC prevents the degradation of securin and mitotic cyclins, thus delaying cell cycle progression in mitosis (Sudakin et al., 2001; Lau and Murray, 2012).

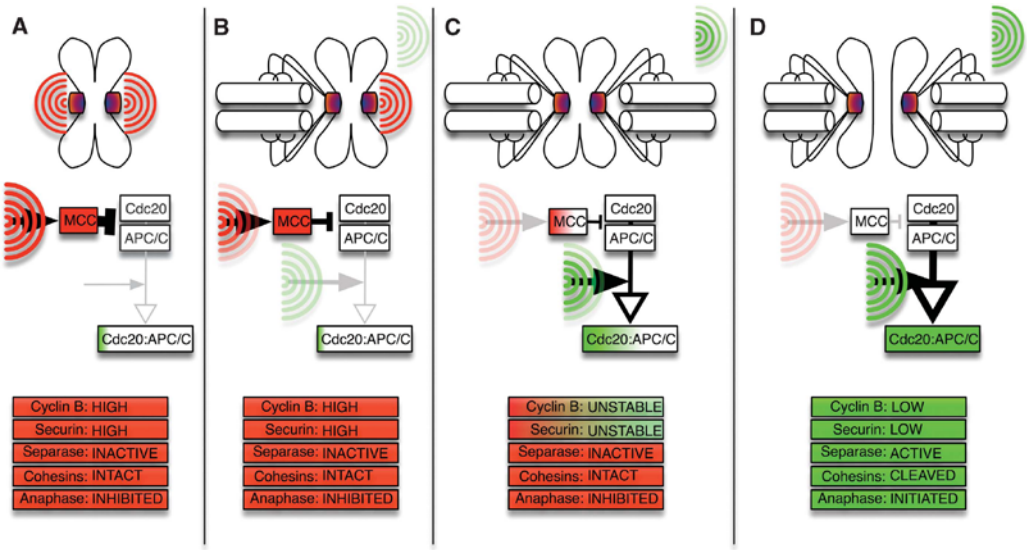


Fig. 3. The Spindle Assembly Checkpoint (Ciliberto and Shah, 2009)

In 1991 two independent genetic screenings identified several genes that are required for cell viability after treatment with microtubule depolymerizing drugs, the *MAD* genes (*MAD1, MAD2, MAD3*) and the *BUB* genes (*BUB1, BUB3*) (Hoyt et al., 1991; Li and Murray, 1991).

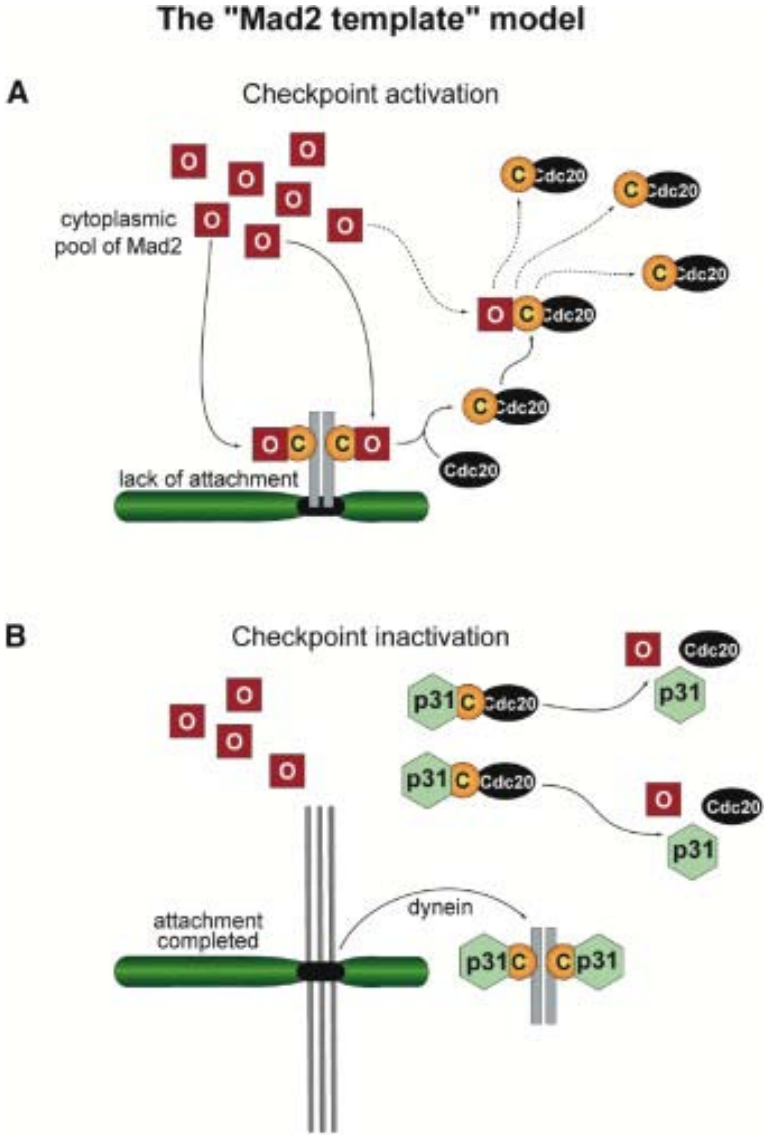


Fig. 4. Mechanism for activation and inactivation of the SAC (Antoni et al., 2005)

It was later shown that Mad2, Mad3 and Bub3 interact to form a Mitotic Checkpoint Complex (MCC) that binds Cdc20 and inactivates the APC/C (Sudakin et al., 2001). Structural analysis has demonstrated that Mad2 exist in two conformational states, called open and closed (O-Mad2 and C-Mad2) (Luo

et al., 2000; Sironi et al., 2002). Mad2 binds tightly to Mad1, which is responsible for its localization at kinetochores during prometaphase; this interaction forces Mad2 to adopt the closed conformation (Chen et al., 1999; Chung and Chen, 2002; Antoni et al., 2005). Moreover, Mad1 bound to C-Mad2 accounts for the more stable pool of Mad2 to the kinetochore. Cdc20 can bind C-Mad2 as well, promoting again its conformational change, leading to the formation of a C-Mad2/Cdc20 complex. O-Mad2 can then bind to C-Mad2-Mad1 and C-Mad2-Cdc20 complexes (Luo et al., 2000; Sironi et al., 2002), where it switches conformation. The formation of these two structures creates a positive-feedback loop that generates new C-Mad2-Cdc20, sequestering the Cdc20 pool and amplifying the signal (Fig. 4). This mechanism explains how a single unattached kinetochore can promote SAC activation and induce a cell cycle delay (Simonetta et al., 2009).

The attachment between mitrotubule and kinetochore is not the only factor that promotes SAC activation, since also tension plays an important role. High and correct kinetochore tension is generated by bipolar attachment; on the other hand, when kinetochores are attached to the wrong pole, a low tension is present. Kinetochore attachment to the wrong pole is destabilized by phosphorylation events carried out by Ipl1 (Aurora B in higher eukaryotes), the catalytic subunit of the chromosome passenger complex that resides at centromeres (Kang et al., 2001; Shang et al., 2003; Cheeseman and Wilson-Kubalek, 2006; Pinsky et al., 2006). This destabilization produces an unattached kinetochore that can be then sensed by the MCC leading to cell cycle arrest. When kinetochores are correctly under tension, they are pulled away from the centromere, so that Ipl1 cannot destroy the attachment

between microtubules and the kinetochore, hence effectively promoting bi-orientation (Tanaka et al., 2002; Keating et al., 2009; Liu et al., 2009).

Once all the kinetochores are attached and under the correct tension, the SAC needs to be inactivated, and several mechanism seem to contribute to this. In particular, in human cells a protein call $p31^{comet}$ is able to break the positive feedback loop based on C-Mad2 (Xia et al., 2004).

In budding yeast, another checkpoint called Spindle Position Checkpoint (SPOC) ensures the fidelity of genome segregation, controlling the correct positioning of the mitotic spindle along the mother-bud axis (Fig. 5) (Stearns, 1997; Fraschini et al., 2008).

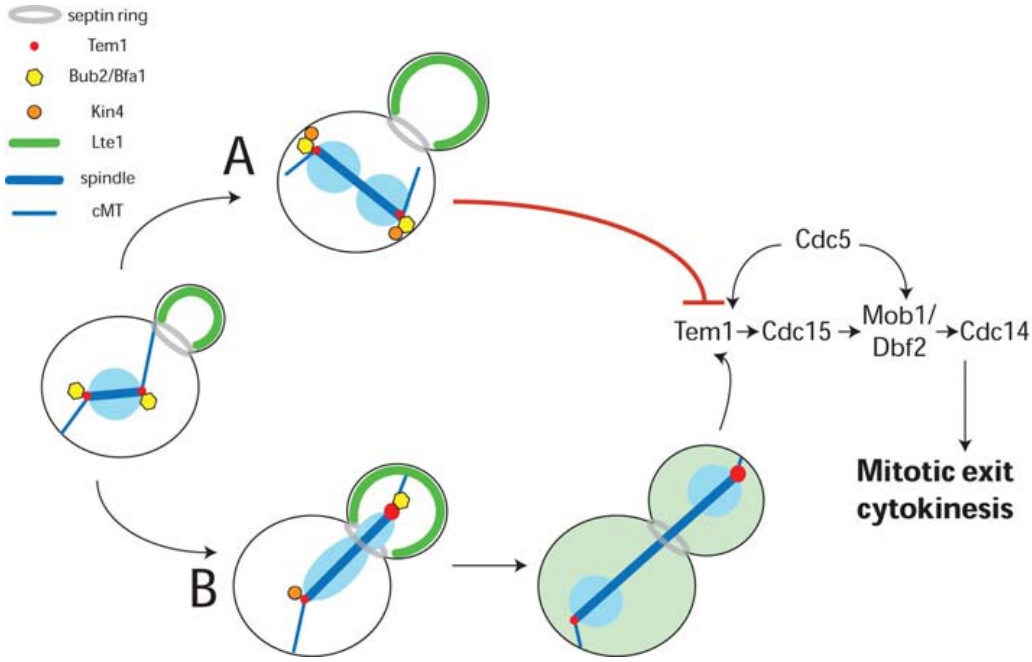


Fig. 5. The Spindle Position Checkpoint (Fraschini et al., 2008)

In *S. cerevisiae* the plane of cell division is established early in the cell cycle, in late G1 phase, and it overlaps with the bud neck. As a consequence, the

mitotic spindle must be aligned orthogonally to the bud neck in order to segregate one genome in the mother and the other in the bud. The SPOC monitors the correct alignment of the spindle along this axis (Fraschini et al., 2008).

In yeast, the positioning of the mitotic spindle is mediated by two different mechanisms, the Kar9-Bim1 and the dynein pathways. Disruption of one of these two mechanisms will lead to low levels of spindle mispositioning, while elimination of both results in the inabilityl to correctly position the spindle and causes cell lethality (Li et al., 1993; Miller and Rose, 1998; Lee et al., 2000). In case of wrong positioning, the SPOC delays cell cycle progression to provide cells with more time to try to realign the mitotic spindle. In fact, mutants defective in the Kar9 or the Dyn1 pathway depend upon the SPOC for survival (Pereira et al., 2000).

Once active, the SPOC delays mitosis by inhibiting the MEN apical factor Tem1 (Geymonat et al., 2002; Nelson and Cooper, 2007). As already mentioned, Tem1 is a GTPase regulated by a GAP complex formed by Bub2 and Bfa1. To trigger the MEN, the GAP complex must be inhibited through a phosphorylation by the polo kinase Cdc5 (Geymonat and Spanos, 2003). Bub2-Bfa1 are also regulated by two different and antagonistic mechanisms involving the Kin4 and Lte1 kinases. The first one is localized at the mother cortex and at the SPBs, where phosphorylates Bfa1 preventing its phosphorylation by Cdc5 and ensuring a high activity of the GAP complex(Chan and Amon, 2009; Falk et al., 2011). The SPB-bound Bfa1 can thus be inhibited by Cdc5 only when the SPB leaves the mother and enters the bud. Lte1, is normally found in the bud. It binds Kin4 kinase on the daughter SPB inactivating it (Bertazzi et al., 2011). Moreover, through a mechanism not yet

understood, Lte1 promotes dissociation of Bub2-Bfa1 from the SPB that remained in the mother cell ,eliminating all the active pool of the GAP complex and allowing a correct exit from mitosis (Geymonat et al., 2009; Bertazzi et al., 2011; Falk et al., 2011). In cells where the spindle is misaligned within the mother, Kin4 is free to bind both SPBs; it phosphorylates Bfa1 keeping the GAP complex active. In this way, Tem1 cannot promote the MEN cascade and the cell cannot exit from mitosis (Fraschini et al., 2008; Geymonat et al., 2009; Bertazzi et al., 2011; Falk et al., 2011).

Interestingly, the SPOC pathway was was initially described only in *S. cerevisiae*, but recent work has opened the possibility that similar pathways may be also present in any cell that needs to divide asymmetrically, like stem cells (Pereira and Yamashita, 2011).

**Polarized growth**

Cell polarization is a fundamental prerequisite for many biological processes in both unicellular and multicellular organisms, such as division, development, directional cell migration and differentiation. Polarization is directed by specific spatial hints provided by the environment or by cell history (Drubin and Nelson, 1996). These cues are the starting point for a signalling that has its main target in the actin cytoskeleton, which undergoes specific rearrangements leading to different polarizations.

Because of its simple genetics, *S. cerevisiae* was the ideal subject?model organism for the detailed characterization of these processes. During the cell cycle, budding yeast goes through polarized growth in order to promote bud formation and extension/expansion/enlargement and subsequently to direct

the correct segregation, toward the bud, of all the elements (proteins, organelles etc) necessary for its survival (Pruyne and Bretscher, 2000).

The yeast actin cytoskeleton is organized into four biochemically and morphologically distinct structures that are: patches, cables, the polarity cap and the cytokinetic ring.

Cortical patches formation and organization are regulated by cortical patch-like proteins structures that include the Arp2/3 complex. These proteins are involved in a mechanism that control polymerization and recycling of actin filaments networks (Goley and Welch, 2006). However their precise role is not yet clear.

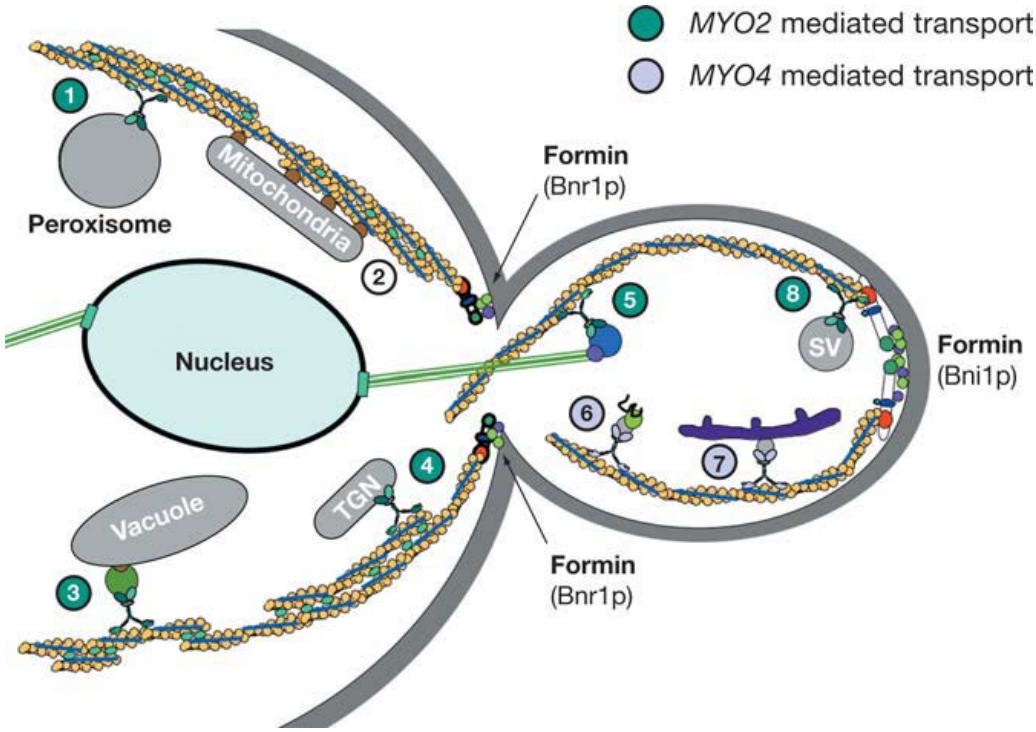


Fig. 6. Localization and action of formins in yeast (Pruyne et al., 2004)

Actin cables are linear bundles of F-actin that are nucleated and elongated by an evolutionary conserved family of proteins called formins (Zigmond, 2004). Budding yeast has two formins, encoded by *BNI1* and *BNR1* (Pruyne et al., 2002).

Each formin contains a C-terminal FH1 and FH2 domain, which is responsible for actin nucleation activity *in vivo* and *in vitro*, and an N terminal rho-GTPase-binding domain (Kikyo et al., 1999; Pruyne et al., 2002). They promote nucleation of actin cables, generating the wires by which the cellular polarization is drawn (Sagot et al., 2001, 2002; Zigmond, 2004).

When yeast cells are unbudded and depolarized, the two formins are randomly diffused. Conversely, when polarization is set up, the positioning of formins establishes the polarized arrays of cables used by the cell for growth. Bni1 localizes at the site of the enlarging bud; its positioning depends upon Spa2, a protein of the polarity cap (Sheu et al., 1998). Bnr1 on the other hand accumulates at the bud neck, in a Gin4 septin–associated kinase dependent manner (Buttery et al., 2007, 2012; Gao et al., 2010). Bni1 promotes actin cables elongation from the bud tip along the bud, while Bnr1 nucleates cables from the neck in the mother cell (Fig. 6). It is unknown how these proteins reach the proper sites, but it seems that a phosphorylation events may be involved in (Sheu et al., 1998; Goehring et al., 2003; Buttery et al., 2012); moreover a recent study demonstrates that at the time of mitotic exit both proteins change their localization in a Cdc14-dependent manner, in order to allow the final depolarization (Bloom et al., 2011).

Another protein important for *in vivo* actin cable assembly, is Bud6/Aip3, which was identified in a two-hybrid screen for proteins interacting with actin (Amberg et al., 1997). Yeast strains defective for Bud6 show inefficient nuclear

migration and nuclear division, defects in the organization of the secretory system, and abnormal septation; all these defects correlate with the impairment in the organization of the actin cytoskeleton (Amberg et al., 1997). Bud6 physically interacts with both Bni1 and Bnr1 and its localization at the bud neck and at the bup tip depends upon the two formins (Kikyo et al., 1999; Delgehyr et al., 2008). Recent studies demonstrate that Bud6 is an actin nucleation-promoting factor that stimulates *in vivo* and *in vitro* the activity of the two formins (Moseley and Goode, 2005; Graziano et al., 2011). Interestingly, it has been recently shown that Bud6 has also a role in priming of the polarity needed for the correct segregation of the mitotic spindle (Fig. 7 (Segal et al., 2000, 2002; Huisman and Segal, 2005; Haarer et al., 2007; Delgehyr et al., 2008; Ten Hoopen et al., 2012). In fact, Bud6 associates with tubulin *in vitro* and contributes to the cortical capture of the astral microtubules at the bud cortex (Segal et al., 2002; Delgehyr et al., 2008; Ten Hoopen et al., 2012); microtubule capture by the bud neck-fraction of Bud6 ensures a mother-bound fate for the new SPB (Segal and Bloom, 2001; Huisman and Segal, 2005). Altogether these evidences suggest that Bud6 may be a mediator between actin and microtubule polarization.

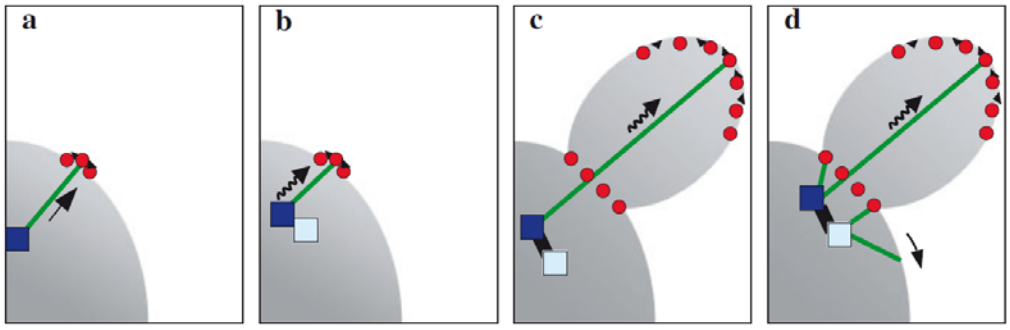


Fig. 7. Model for Bud6-microtubules capture (Bud6 is represented as red dots; the two SPBs are displayed as blue squares (dark blue dSPB, light blue mSPB)) (Huisman and Segal, 2005)

**Haspin**

Haspin (*H*aploid Germ Cell-*s*pecific nuclear *P*rote*in* Kinase) is the product of a gene called *GSG2* that was originally identified in mouse testis. Originally haspin was proposed to have a role in cell-cycle cessation and differentiation of haploid germ cells (Tanaka et al., 2001). Orthologues of this gene were found in all eukaryotes from yeast to plants or flies (Higgins, 2001, 2003).

Haspin is an atypical serine/threonine kinase (Eswaran et al., 2009; Villa et al., 2009). In the last decade human haspin has been proposed to play a fundamental role in the regulation of mitosis. It localizes on condensed chromosomes, particularly at centromeric regions and its depletion prevents normal chromosome alignment at the metaphase plate (Dai et al., 2005, 2006). In particular, it was shown that in metaphase haspin phosphorylates threonine 3 in the histone H3 (Dai et al., 2006). This modification provides the proper recruiting site for the chromosome passenger complex (CPC) at inner kinetochores. The localization of the CPC is a prerequisite for successful chromosome segregation (Kelly et al., 2010; Wang et al., 2010; Yamagishi et al., 2010). Moreover, haspin has been shown to be a direct substrate of the catalytic subunit of CPC, Aurora B kinase, that in this way facilitates phosphorylation of H3-Thr3, therefore promoting its own accumulation at the inner kinetochore (Wang et al., 2011). Haspin has been also implicated in the maintenance of chromosome cohesion (Dai et al., 2006). This loss of cohesion caused by haspin depletion compromises spindle-pole integrity, as an indirect consequence of the failure to properly integrate chromosome- and centrosome-initiated pathways for spindle formation (Dai et al., 2009).

The role of haspin has been studied in *A. thaliana* and in the yeast *S. pombe* (Yamagishi et al., 2010; Ashtiyani et al., 2011). In particular, evidences observed in *A. thaliana* led to the hypothesis that, beyond its role in histone H3 phosphorylation, haspin may play a role in plant development (Ashtiyani et al., 2011).

In *Saccharomyces cerevisiae* two haspin homologues, encoded by *ALK1* and *ALK2,* have been described, but their function is still unknown.

A preliminary study shows that these proteins are not expressed in G1, and their levels peak in mitosis and late S/G2 phase, respectively. Furthermore, both proteins are hyperphosphorylated during mitosis (Nespoli et al., 2006).

The levels of Alk1 and Alk2 are regulated through transcriptional and post-translational mechanisms. Yeast haspin stability is controlled by "KEN" and "destruction" boxes present in their amino acid sequences. These motifs were originally identified in cyclins and were found to be responsible of their ubiquitin-mediated degradation (Nespoli et al., 2006). Neither Alk1 nor Alk2 seem to be involved in histone H3-Thr3 phosphorylation, while they exhibit autokinase activity *in vitro*. Finally, overexpression of *ALK2*, but not of *ALK1*, causes a mitotic arrest, which correlates with the kinase activity of the protein (Nespoli et al., 2006).

The analysis of haspin role in *S. cerevisiae* is therefore only at the beginning. Moreover, since in this organism haspin seems not involved in the recruitment of Aurora B homologue Ipl1, budding yeast is a perfect model for the investigation of new haspin functions that are independent from histone h3 phosphorylation.

# Aim of the Project

The segregation of the genetic material is one of the key steps during the cell cycle. Errors in this process lead to aneuploidy that is a precursor of cancer. Moreover, also the correct partitioning cellular structures before cytokinesis can be extremely important, in particular for cells that undergo asymmetric cell division (ACD), in which a cell generates daughters with distinct characteristics, for example during embryonic development or stem cell homeostasis. For this purposes, cells have developed an intricate network of processes that organize and control the accuracy of the segregation.

Haspin is a protein kinase conserved throughout evolution, whose role has been recently established in human cells (Higgins, 2010; Wang et al., 2010), in the yeast S. pombe (Yamagishi et al., 2010) and in the plant A. thaliana (Ashtiyani et al., 2011).

These works have demonstrated that haspin phosphorylates histone H3-Thr3 creating a docking site for the recruitment of the chromosomal passenger complex (Kelly et al., 2010; Wang et al., 2010; Yamagishi et al., 2010). This function is required for a correct chromosome congression at the metaphase plate and chromosome segregation. Moreover, it has also been shown that loss of haspin causes defects in spindle pole integrity (Dai et al., 2009) and in

plant embryonic development (Ashtiyani et al., 2011), suggesting that haspin may have multiple roles, still far for being identified. Interestingly, some evidence correlates haspin with development: haspin was first identified in mouse germ cell lines, it is highly expressed in proliferating cells and, in plants, haspin affect embryonic development starting from the first cell division. The role of haspin in budding yeast, on the other hand, was still completely obscure. In budding yeast, in fact, H3-Thr23 is not phosphorylated and the chromosome passenger complex is recruited to the kinetochores through a different mechanism. This suggested that haspin may also have other targets and may work in different processes. Preliminary data obtained in the lab suggested that yeast haspin may be involved in regulating mitotic divisions. Budding yeast lacking H3-Thr3 phosphorylation was a perfect model to investigate new functions for haspin. Moreover these cells are a classical system for studying asymmetric cell division. Indeed, in most eukaryotic cells the cell division plane is established late in the cell cycle so that it is setup orthogonally to the mitotic spindle, and it splits the cell in half. In budding yeast, on the other hand, cells establish first the position of the plane of division, in late G1 phase, and only after the spindle is properly aligned and positioned so that it will be orthogonal to the cell division plane. Cytokinesis will then generate two unequal cells.

In this work we have investigated the mitotic role of yeast haspin and have uncovered a new function in regulating the coordination between mitosis and cellular polarization. Finally we have also demonstrated that haspin plays a similar role in cellular polarization also in human cells. These studies lead to the hypothesis that haspin may have a relevant function in controlling asymmetric cell division.

# Main Results

In human and in *S.pombe* cells, haspin kinase phosphorylates histone H3-Thr3, and this allows the recruitment of the catalytic subunit of the Chromosomal Passenger Complex (CPC), Aurora B, at the kinetocore (Kelly et al., 2010; Wang et al., 2010; Yamagishi et al., 2010). This step is a prerequisite for a successful chromosome segregation. However, haspin depletion in human cells causes also other phenotypes, involving centrosome integrity (Dai et al., 2009) and cell division (Ashtiyani et al., 2011). This indicates that further studies are required to elucidated the exact role(s) of the kinase and the possible novel target(s) of its function.

In budding yeast two haspin ortologue are present, *ALK1* and *ALK2*, but their functions have not been defined yet. The high conservation of the kinase domain, the absence of H3-Thr3 phosphorylation and the characteristics of budding yeast (simple genetics, sequenced genome, particular morphology) make this organism a good candidate to better investigate in detail the role of haspin in mitosis.

We discovered that *Sc*Haspin is involved in a pathway that coordinates cellular polarization with mitosis. Loss of this function leads to nuclear missegregation and cell lethality.

After a transient mitotic arrest, in cells deleted for *ALK1* and *ALK2* many polarization factors are mislocalized, and accumulate in the daughter cell; in particular, many components of the polarisome complex show an evident unbalancing toward the bud and some cases even a strong accumulation at the bud tip. Among these factors is Bnr1, a Diaphanous-related formin responsible for the nucleation of actin cables, the stuctures that govern cellular polarization. Bnr1 is normally restricted to the bud neck, where it nucleates actin cables directed to the mother cell; in *alk1*Δ*alk2*Δ mutants arrested in mitosis, beside the bud neck localization, Bnr1 is also accumulated at the bud tip. The polarisome component Bud6 also shows an aberrant localization: it disappears from the bud neck and accumulates in the bud. The mislocalization of these two important factors, and the fact that Bud6 promotes actin cable nucleation by the formin, can easily explain why in *alk1*Δ*alk2*Δ cells the actin component of the cytoskeleton leaves the mother and is confined within the bud, which does not stop its growth and becomes larger than the mother. This view is reinforced by the fact that disrupting the septin complex, a ring positioned at the bud neck that helps maintaining the partition of polarity factors between mother and bud, is sufficient to rescue the mislocalization of polarity factors in *alk1Δalk2Δ* cells and to restore proper nuclei segregation.

The accumulation of polarity factors and actin within the bud causes the mitotic spindle to be strongly pulled toward the bud so that, in anaphase, it elongates and segregates the two nuclei within this single cell compartment, generating an anucleated mother cell and a binucleated daughter that won't be able to survive.

We have correlated this defect with different kinds of mitotic arrest, concluding that in any case in which cells need to delay anaphase (for example when microtubule attachments to kinetochores have not been successfully achieved), haspin contributes to reorganize the polarization factors so that proper chromosome segregation can be completed.

Finally, we obtained preliminary evidence suggesting that this new haspin function is evolutionary. Indeed, both human and *A. thaliana* haspin-coding genes fully complement the polarity defects observed in *alk1*Δ*alk2*Δ yeast mutants, suggesting a conservation of targets.

To extend this evidence we investigated the effects of haspin depletion in human cells. HeLa epithelial cells represent a useful model to investigate the mechanisms governing spindle orientation. In fact, in appropriate culture conditions, HeLa cells position the mitotic spindle orthogonal to the adhesion plane; we found that, in this situation, depletion of haspin leads to misalignment of the mitotic spindle, causing cells to divide along a wrong axis. This is likely due to an alteration in actin distribution that, in absence of haspin, loses its cortical localization, removing the cues necessary for a correct spindle alignment.

# Conclusions and Future Prospects

In this study we reveal a new function for the evolutionary conserved haspin kinase. We describe a role for this protein in modulating the dynamics that coordinate mitotic events and the polarization program of the cell. We demonstrate that haspin controls the localization of different polarization factors. The accumulation of such polarity cues in the bud promote spindle elongation within this cellular compartment leading to nuclear missegregation and aneuploidy.

The coordination between mitosis and polarity factors is of critical importance for those processes, such as embryonic development and stem cells differentiation, that are based on asymmetric cell division.

The precise mechanism through which haspin controls this dynamics is still not clear. The identification of the direct targets of the kinase will be essential to unambiguously determine its role and to clearly understand these fundamental pathways. For this purpose the use of high-throughput approaches has the potential to yield large amounts of information covering several pathways in which haspin may potentially be involved. In particular we are planning a mass-spectrometry analysis for the identification of peptides directly phosphorylated by the kinase, and a screening for synthetic lethality

that may help in identifying genetic interactors, allowing pathway reconstruction.

In a parallel strategy, we plan to pursue a candidate approach to clearly determine the mechanism involving haspin role in the establishment of cell polarity and cell division axis in mammalian cells. This may yield important hints into the mechanism modulating stem cell homeostasis, ACD and cancer development.

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# Acknowledgements

# Part II

**Yeast haspin kinase regulates polarity cues necessary for mitotic spindle positioning, and is required to tolerate mitotic arrest**

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Running title: Haspin kinase and mitotic cell polarity

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**Abstract**

Haspin is an atypical protein kinase that in several organisms phosphorylates histone H3 on Thr3 and is involved in chromosome segregation. In *Saccharomyces cerevisiae,* H3Thr3 phosphorylation has never been observed and the function of haspin is unknown. We show that deletion of *ALK1* and *ALK2* haspin paralogs causes the mislocalization of polarisome components. Following a transient mitotic arrest, this leads to an overly polarized actin distribution in the bud and the mitotic spindle is pulled in the bud. Here, it elongates generating anucleated mothers and binucleated daughters. Reducing the intensity of the bud-directed pulling forces partially restores proper cell division. We propose that haspin controls the localization of polarity cues to preserve the coordination between polarization and the cell cycle, and to tolerate transient mitotic arrests. The evolutionary conservation of haspin and of the polarization mechanisms suggest that this function of haspin is likely shared with other eukaryotic cells, where haspin may regulate asymmetric cell division.

Key words: haspin, budding yeast, spindle orientation, polarization, mitosis

**Introduction**

Asymmetric cell division is a fundamental process in stem cell biology and development. The appropriate balance between symmetric and asymmetric divisions is important to expand and maintain the stem cells pools, and to generate progenitors for differentiation. Disruption of this balance may lead to cancer (Gonzalez, 2007; Knoblich, 2010; Wodarz and Näthke, 2007). One essential step for asymmetric division is the proper distribution of polarization factors within the cell; this drives the unequal partition of cellular components. Moreover, the establishment of a correct cell division axis also relies on cell polarity cues, and is connected to the orientation of the mitotic spindle (Siller and Doe, 2009; Gönczy, 2008). Regulated polarization and spindle orientation

are crucial in both unicellular and multicellular organisms, where they are involved in fundamental processes, such as epithelial polarization, asymmetric cell division, morphogenesis, organogenesis and development (Clevers, 2005; Quyn et al., 2010; Gray et al., 2010; Tanos and Rodriguez-Boulan, 2008). The molecular mechanisms underlying spindle orientation are still largely unknown. Budding yeast has been used to study spindle positioning and asymmetric cell division. Indeed, after cytokinesis daughter cells have different sizes. The cell division plane is established early in the cell cycle and before assembly of the mitotic spindle (Etienne-Manneville, 2004; Fraschini et al., 2008; Segal and Bloom, 2001), therefore, a surveillance mechanism called spindle position checkpoint (SPOC) oversees spindle positioning and delays mitotic exit and cytokinesis in case of errors (Bardin et al., 2000; Lew and Burke, 2003; Nelson and Cooper, 2007; Pereira et al., 2000). A similar checkpoint has been recently described in *Drosophila* (Cheng et al., 2011), suggesting that these mechanisms exist in various multicellular eukaryotes. Haspin is an atypical protein kinase, highly conserved throughout evolution (Higgins, 2001). The budding yeast genome codes for two haspin paralogs, Alk1 and Alk2, whose protein levels peak in mitosis and late-S/G2, respectively, and are modulated by the anaphase-promoting complex APC. Both proteins are phosphorylated in mitosis, albeit the physiological significance of this modification is still unclear. Finally, overexpression of *ALK2* prevents mitotic progression, suggesting that a precise regulation of haspin is critical for cell division (Nespoli et al., 2006). In mammalian cells, haspin knock-down arrests cells in mitosis and prevents proper chromosome congression at the metaphase plate (Dai et al., 2005; Dai et al., 2006); moreover, haspin is responsible for histone H3Thr3 phosphorylation, which is involved in the recruitment of the chromosomal passenger complex (Wang et al., 2010; Kelly et al., 2010; Yamagishi et al., 2010). In *A. thaliana*, haspin is also involved in plant development; indeed, it contributes to embryonic patterning (Ashtiyani et al., 2011) However, the precise function of haspin is still not fully understood and it is likely that more kinase targets exist,

particularly in budding yeast, where phosphorylation of H3-Thr3 has never been observed. Here we show that in the absence of haspin, budding yeast cells fail to properly recruit polarity factors that are important for the establishment of a balance in the forces acting on spindle positioning. If mitosis is prolonged in these cells, the spindle is pulled within the daughter cell and nuclear division generates an anucleated mother and a binucleated daughter. Haspin is thus essential to tolerate a transient mitotic arrest and for the maintenance of the coupling between polarization and cell cycle progression. Our findings may help explaining the developmental defects observed in *Arabidopsis* haspin mutants, and its evolutionary conservation suggests that haspin may be important for the proper positioning of polarity factors also in other eukaryotic cells and may be a key player in the control of asymmetric cell division.

## Materials and Methods

### Yeast strains and plasmids.

All strains used in this study are isogenic to W303, and are listed in Table S1. Standard conditions for yeast cell cultures have been previously described (Rose et al., 1990). Standard molecular genetics techniques were used to construct plasmids and strains. The centromeric plasmids containing *GFP-BUD6* and *GFP-BNR1* under their endogenous promoter were kind gifts of M. Segal (Segal et al., 2000a) and D. Pellman (Buttery et al., 2007). PCR-based genotyping was used to confirm gene disruption and tagging. Gene overexpression under the inducible *GAL1* promoter was achieved by adding 2% galactose to raffinose-containing medium. Temperature-sensitive mutants were grown either at permissive (25°C) or at restrictive temperature (37°C).

### Spindle assembly checkpoint assays.

To analyze Pds1-HA stability during nocodazole treatment, cells were grown in YPD medium, synchronized in G1 with α-factor (2 μg/ml), released in presence

of nocodazole (10 μg/ml). At different times, samples were collected to obtain total protein extracts that were resolved by SDSPAGE and analysed by western blotting using anti-HA antibodies (12CA5), as previously described (Sabbioneda et al., 2007). To evaluate sister chromatid cohesion, cells were grown as described above in medium containing adenine and samples taken at different time-points were fixed with ethanol and GFP visualised by fluorescence microscopy with a Leica DMRA2 equipped with CCD camera (Leica DC 300F). Images were processed with Leica FW 4000 software.

**Actin staining.**

Cells were grown as described, fixed with formaldehyde (3,7%) and washed twice with water and once with PBS. After incubation for 45 min with Alexafluor488-coniugated phalloidin, actin was visualized by fluorescence microscopy as described above.

**Concanavalin A staining.**

Cells were grown in YPD and synchronized with α-factor (2 μg/ml). After G1 arrest cells were washed with PBS and resuspended in 125μl of Alexa fluor 594-conjugated Concanavalin A at the concentration of 40μg/ml in the dark at room temperature. After 10 min, cells were washed and resuspended in the appropriate medium, as indicated.

**Determination of incorrect anaphase.**

Cells were synchronized in G1 and released in nocodazole, as described above. After 150 min in nocodazole, cells were released in fresh medium without the drug. At the indicated times after removal of nocodazole, cells were fixed with formaldehyde (3,7%) and *in situ* immunofluorescence was performed as described previously (Fraschini et al., 1999). Tubulin was visualized with anti-tubulin antibodies (YOL34, 1:150) followed by indirect immunofluorescence with Alexafluor594conjugated donkey anti rat Ab (1:1000), while DNA

staining was performed with DAPI. For live-cell imaging, cells were adhered onto Fluorodishes (World Precision Instruments) and covered with SD medium. Cells were imaged at 30°C using a 63X immersion objective mounted on a Zeiss LSM780 confocal microscope controlled by the Zen2010 software. Eight Z stack images with an optical section spacing of 0.7 µm were acquired every minute for two hours with a laser power of 0.4%. Z-stacks were max-projected by ImageJ.

**FRAP analysis**

FRAP experiments were performed as previously described (Dobbelaere et al., 2003) on a Zeiss LSM 510 confocal. Briefly logarithmically growing cells expressing GFP-Cdc12 were grown overnight in YEPD, treated with nocodazole 10µg/ml for 150 min, then resuspended in synthetic complete medium and spread on 2% agar pads. Half the septin ring was bleached with a sequence of 20 to 25 irradiations at 50% of laser intensity. Fluorescence intensities were analyzed with ImageJ. Background staining in each cell was subtracted. To correct for general bleaching, fluorescence intensities of septin rings were normalized to those of 2–3 reference cells.

**Results**

**Haspin defective cells are sensitive to microtubule depolymerizing drugs**.

In human, *A. thaliana* and *S. pombe* cells, haspin phosphorylates H3Thr3 and is important for proper function of the chromosome passenger complex; loss of haspin results in chromosome congression defects and loss of spindle-pole integrity (Dai et al., 2009; Wang et al., 2010; Yamagishi et al., 2010; Ashtiyani et al., 2011). No evidence for a similar function in budding yeast exists; consistently, histone H3 does not seem to be phosphorylated at Thr3 in *S. cerevisiae*. Deletion of *ALK1* or *ALK2*, either single or combined, does not cause any immediately apparent phenotype (Nespoli et al., 2006). Overexpression of *ALK2* delays the onset of anaphase (Nespoli et al., 2006),

suggesting that proteins other than histone H3 may be targets of haspin in mitosis. To understand haspin function in budding yeast mitosis, we analyzed the sensitivity to microtubuledepolymerizing drugs (i.e. benomyl or nocodazole) of cells deleted for the genes coding for the two haspin paralogues. As shown in Figure 1A, single *alk1*Δ or *alk2*Δ mutant cells show, respectively, mild or no sensitivity to benomyl. However, the viability of *alk1*Δ*alk2*Δ double mutant cells is heavily compromised in the presence of the drug, similarly to what observed in strains lacking the *MAD2* gene, a central factor in the spindle assembly checkpoint (SAC) elicited by microtubule disassembly. Loss of haspin causes cell lethality also after a transient treatment of a liquid culture with nocodazole (Figure S1A). Similar phenotypes were observed in a kinase deficient *alk1-kd alk2-kd* double mutant (Nespoli et al., 2006) (Figure 1B), indicating that survival following benomyl treatment relies on the kinase activity of haspin. Benomyl and nocodazole have three major effects: depolymerize microtubule, activate the SAC, causing a mitotic delay. The lethality described above could be explained if loss of haspin caused a SAC defect. The anaphase inhibitor securin (Pds1 in *S. cerevisiae*) prevents sister chromatids separation and is stabilized by SAC activation, resulting in cell cycle arrest in metaphase. On the contrary, SAC defective mutants degrade securin, separate sister chromatids and exit mitosis, despite disassembly of the mitotic spindle by nocodazole treatment (Musacchio and Salmon, 2007; Peters, 2006). To verify the state of the SAC in haspin mutants, we followed the fate of Pds1 in synchronous cultures released from G1 into nocodazole-containing medium. As shown in Figure 1C, in *alk1*Δ*alk2*Δ double mutant cells Pds1 is stable after nocodazole treatment, similarly to what observed in wild-type cells. On the contrary, in SAC-defective *mad2*Δ cells, Pds1 is rapidly degraded after nocodazole treatment. In agreement with this finding, visualization of chromosome V arms with the tetO/TetR-GFP system (Michaelis et al., 1997), shows that upon nocodazole treatment sister chromatids remain cohesed in *alk1*Δ*alk2*Δ mutants and in wild-type cells, whereas they dissociate in the

absence of Mad2 (Figure 1D), indicating that in budding yeast loss of haspin does not affect SAC activation after microtubule depolymerization.

**Loss of haspin causes spindle mispositioning and nuclear missegregation after mitotic arrest.**

The benomyl sensitivity of *alk1*Δ*alk2*Δ cells could derive from a failure to reassemble a spindle or to properly position the reassembled spindle after microtubule depolymerization. We tested the kinetics of mitotic spindle rebuilding and orientation, as well as of nuclear division, in synchronous wild-type and *alk1*Δ*alk2*Δ mutant cells after release from a nocodazole treatment (Figure 2 and Figure S1B). As shown in Figure 2A, 60 min after the removal of the drug wild-type cells rebuild a mitotic spindle that elongates along the mother-bud axis; by 100 min after the release the genome is fully segregated between mother and daughter cell. Strikingly, in *alk1*Δ*alk2*Δ mutant cells, while a bipolar spindle is fully reassembled 60 min after nocodazole removal, it is not aligned along the mother-bud axis, but remains confined within a single cell compartment where it then elongates exhibiting an aberrant curved shape (Figure 2A). By the 100 min time-point virtually 100% of *alk1*Δ*alk2*Δ anaphase cells contain two nuclei within a single cell body, while this aberrant population remains below 5% in wild-type and below 20% in *alk1*Δ or *alk2*Δ single mutant strains (Figure 2B). A kinase deficient *alk1-kd alk2-kd* double mutant strain exhibits a similar phenotype (Figure S1C), indicating that haspin kinase activity is important for proper positioning of the mitotic spindle following microtubule depolymerization. We investigated whether spindle misalignment in *alk1*Δ*alk2*Δ cells was due to alterations in spindle reassembly or to spindle positioning. After a transient nocodazole treatment, we analysed by time lapse video microscopy, cultures of wild-type and *alk1*Δ*alk2*Δ cells (Movies S1 and S2). The data show that in haspin-deleted cells the bipolar spindle is rebuilt with essentially wild-type kinetics (Figure S1B, but exhibits a clear positioning defect, resulting in nuclear division within an individual cell body (Figure 2C).

It is interesting to note that nuclear division always takes place in the largest cell body, which surprisingly corresponds to the bud (see below).

In budding yeast, spindle mispositioning is detected by the spindle position checkpoint (SPOC), which prevents mitotic exit when the spindle is not correctly aligned along the mother-bud axis (Bardin et al., 2000). Surprisingly, we noticed that, after removal of nocodazole in *alk1*Δ*alk2*Δ cells the nucleus divides (Figure 2A), the misaligned spindle is timely disassembled (see Movie S1 and S2) and a new round of DNA replication follows (Figure S1D). These data indicate that, after transient metaphase arrest, loss of haspin leads to spindle misalignment without SPOC activation. To further characterize this phenotype, we investigated whether in *alk1*Δ*alk2*Δ cells the spindle is elongated in the mother, in the bud or randomly in both cellular compartments. To unequivocally distinguish mothers from daughters we took advantage of a previously described simple assay (Ross and Cohen-Fix, 2004). Exponentially growing cells are treated with α-factor, which arrests cells in G1 with a recognizable mating projection (shmoo). The removal of α-factor allows cells to proceed in the cell cycle and shmooed mothers can be easily distinguished from round daughter cells. We released αfactor G1-arrested cells in nocodazole-containing medium. After 150 min, all cells were arrested in metaphase with disassembled mitotic spindles. Cultures were then released in fresh medium to allow spindle reassembly and, 60 min after the release from nocodazole, samples were fixed and stained with DAPI. Fluorescence microscopy analysis of *alk1*Δ*alk2*Δ cultures shows that in ~75% of the cases rounded binucleated and shmooed anucleated cells are detected, indicating that nuclear segregation occurs almost exclusively in the bud (Fig 2D). This result was further confirmed by staining mothers with Concanavalin A before the arrest. Figure 2E shows that, in cells lacking haspin, the mitotic spindle elongates within the unstained compartments (bud). The migration of the spindle into the bud may also explain the failure to trigger the SPOC, which is known to be activated by spindle misalignment within the mother cell (reviewed in Fraschini et al.,

2008). Surprisingly, we noted that when *alk1*Δ*alk2*Δ cells were arrested in metaphase the bud continued to grow, often becoming larger than the mother. The similarity in the kinetics of spindle rebuilding in wild-type and double mutant cells (Movie S1, S2) indicates that Alk1 and Alk2 are not involved in clearing of the drug after nocodazole treatment, or in the proper reassembly of the mitotic spindle. The lethal event, observed after nocodazole treatment, may be due to the prolongation of mitosis. To test this third hypothesis we took advantage of a strain expressing *CDC20,* an activator of the anaphase promoting complex/cyclosome (APC/C), under the control of the *GAL1* promoter. In galactose containing medium *CDC20* is expressed and cells can conclude mitosis; in the presence of glucose, repression of *CDC20* expression causes cells to arrest at metaphase with one replicated nucleus and a short spindle. Cells expressing *GAL1-CDC20* were grown in galactose and arrested in G1 with α factor. Cultures were released in glucose to repress *CDC20* expression, inducing a metaphase arrest. After 210 min *CDC20* was re-induced to allow cells to progress through mitosis. As shown in Figure 2F, G, 60 min after anaphase entry cells have finished mitosis: more than 50% of *alk1*Δ*alk2*Δ mutants cells have divided their nucleus in the bud, completed cytokinesis, and are detectable as unbudded binucleated cells; on the contrary, wild-type cells have divided normally and are mononucleated. These data indicate that when metaphase is prolonged haspin is required to avoid spindle mispositioning and prevent cell lethality. Spindle positioning is driven by two pathways governed by Kar9 and Dyn1, respectively. While cells tolerate the loss of either one of the pathways, elimination of both, in *kar9*Δ*dyn1*Δ cells, is lethal. In order to investigate whether loss of haspin may affect either the Kar9 or the Dyn1 pathway, we crossed *alk1*Δ*alk2*Δ cells with *kar9*Δ or *dyn1*Δ strains. No synthetic lethality was observed and, as shown in figure S2A, elimination of either one spindle positioning pathway in cells lacking haspin does not result in growth defects at temperatures ranging from 25°C to 37°C. This finding implies that haspin does not impair the Kar9 or the Dyn1 spindle positioning

mechanisms. Furthermore, we tested whether the phenotype detected in *alk1*Δ*alk2*Δ cells may be due to a deregulation of Kar9 or Dyn1. Figure S2B shows that neither *kar9*Δ or *dyn1*Δ affect the nuclear missegregation displayed by cells lacking haspin. These results strongly suggest that no direct relationship exists between haspin, Kar9 and Dyn1.

**Haspin is essential for proper organization of cell polarity factors in mitosis.**

The alignment of the mitotic spindle is governed by actin. We thus verified whether deletion of haspin correlated with a defective distribution of actin. In cycling cells, actin is localized at the tip of the growing bud, where it directs polarized growth; once cells reach G2/M and shift to isotropic growth, actin is re-distributed uniformly to both mother and daughter cells (Pruyne and Bretscher, 2000). Wild-type and haspin-deleted cells were arrested in G1 with α-factor to generate shmooed mothers and released in nocodazole to induce metaphase arrest. Actin distribution was then analyzed at various times after nocodazole wash-out. As shown in Figure 3A, in nocodazole-arrested wild-type cells actin is evenly distributed between mother and daughter cells. On the contrary, haspin defective mutants fail to redistribute actin, which in 80% of the cells is largely accumulated in the bud, consistently with the spindle misalignment phenotype, and with the increased bud size observed when these mutants are arrested in mitosis. This result was also confirmed by staining mother cells with Concanavalin A before the mitotic arrest (Figure 3B).

Partitioning of polarity factors is controlled by the septin ring, which acts as a selective barrier at the bud neck (Barral et al., 2000). We thus tested whether disruption of the septin complex would rescue the misdistribution of actin in *alk1*Δ*alk2*Δ cells. We used a *cdc12-6* allele, coding for a temperature sensitive septin subunit, which at non-permissive temperature causes rapid disruption of the septin ring (Barral et al., 2000). a*lk1*Δ*alk2*Δ*cdc12-6* cells were arrested with nocodazole at 25°C for 2,5 hours, shifted to 37°C for 45 min, and released in

YPD at 37°C in the absence of the drug. Figure 3C and 3D show that disruption of the septin ring allows proper redistribution of actin in *alk1*Δ*alk2*Δ cells and rescues the spindle misalignment and nuclear division defect. Since the septin complex undergoes cell cycle dependent changes in its stability, switching from a frozen to a fluid structure (Dobbelaere et al., 2003), such observations may suggest that haspin modulates septin ring stability. We studied the ring dynamics by following the Shs1 component, which is dephosphorylated in the fluid state, and by FRAP analysis. Fig S3 shows that loss of haspin does not grossly affect Shs1 phosphorylation, as detected by SDS-PAGE mobility shift analysis, or septin dynamics, indicating that haspin does not influence the septin ring.

**Haspin is required to maintain the correct balance of the formin-dependent spindle pulling forces.**

Actin distribution is also controlled by formins, evolutionary conserved factors important for cell polarity and morphogenesis. In budding yeast two formins have been identified: Bni1 is found at the bud tip from where it organizes actin cables within the bud, while Bnr1 is present at the bud neck and promotes formation of actin cables within the mother cell. Given the misdistribution of actin caused by deletion of *ALK1* and *ALK2*, we investigated whether Bnr1 localization is compromised by loss of haspin. GFP-Bnr1 expressing cells were arrested in metaphase/anaphase with nocodazole; Figure 4A, B shows that, after release from the mitotic arrest, in wild-type cells Bnr1 is concentrated at the bud neck. Intriguingly, loss of haspin induces an abnormal localization, so that Bnr1 is equally distributed between the bud tip and the bud neck. In haspin-defective cells, the Bnr1 molecules at the bud tip may be responsible for an excessive force driving polarity toward the bud during mitotic arrest, explaining the defective distribution of actin. One prediction is that deletion of *BNR1* may prevent this asymmetry, reduce the pulling forces and correct the defects due to the loss of haspin. Figure 4C, D shows that combining *bnr1*Δ with *alk1*Δ*alk2*Δ

rescues part of the benomyl sensitivity and nuclear division defects caused by lack of haspin activity. Deletion of *BNI1*, encoding the other formin normally localizing at the bud tip, has a similar suppressive effect (Figure 4D). Bnr1, interacting with Bud6, controls its recruitment at the septin ring, which is crucial for the interaction of astral microtubules with the bud neck, and is required for proper spindle alignment (Segal et al., 2000b). We thus investigated whether haspin depletion affected Bud6 localization. Cells expressing GFP-Bud6, were arrested in nocodazole and released in drug-free medium. In wild-type cells, Bud6 is enriched at the bud neck and is also present at the cell periphery, after the removal of nocodazole. Intriguingly, in most *alk1*Δ*alk2*Δ cells Bud6 is mislocalized: it is noticeably absent from the bud neck, and it is evidently accumulated within the bud (Figure 5A, B). The presence of Bud6 at the bud neck is important to establish and maintain a symmetry in the SPBs distribution, which is essential for correct spindle positioning. The finding that in haspin deleted cells Bud6 is missing from the neck and enriched in the bud could explain why the mitotic spindle is restricted to the daughter cell in these mutants. Bud6 is also a member of the polarisome complex, which drives actin cables organization (Park and Bi, 2007). We thus verified the localization of the Spa2 polarisome component. In cells arrested in metaphase/anaphase we observed that lack of haspin forced the polarisome to remain in the daughter cell, while in wild-type cells Spa2 is equally redistributed between mother and daughter (Figure S4). Altogether, these results indicate that loss of haspin causes the accumulation of polarity factors in the bud, resulting in the asymmetric distribution of actin, and generating an unbalance of the forces responsible for spindle alignment. Rebalancing of such forces, through the elimination of either one of the two formins allows the correct positioning of the mitotic spindle.

**Haspin is essential for cell survival after SAC-induced mitotic delay.**

It is somewhat surprising that no clear viability defect is observed in haspin

mutants during an unperturbed cell cycle, given the effects on the localization of polarisome factors and formins. To verify whether haspin controls polarization factors also in a normal cell cycle, we monitored Bud6 localization in *alk1*Δ*alk2*Δ released from a G1 arrest. Figure 6A shows that loss of haspin causes Bud6 to delocalize from the bud neck and accumulate only in the bud also in cycling cells. We could not detect a mislocalization of Bnr1 and actin in these conditions. During an unperturbed cell cycle, the absence of haspin causes a molecular defect (i.e. loss of Bud6 from the bud neck), but it is not sufficient to cause cell lethality. This suggests that in a mitotic arrest, the correct localization of Bud6 is important to preserve the proper position of other polarity cues. We propose that, through haspin kinase, yeast cells coordinate mitotic progression with the regulation of the spindle pulling forces, keeping the SPBs separated in the mother and daughter cell and properly positioning the mitotic spindle. If this is the case, one prediction is that haspin should be important for maintenance of this coordination and survival in cells where mitosis encounters problems and needs to be temporarily delayed, such as when SAC is activated in response to a misassembled mitotic spindle or unattached chromosomes. We exploited two different yeast temperature sensitive (ts) conditional mutations that cause SAC activation and prolongation of mitosis, affecting the structure of the kinetochore (*mif2-3*) or its attachment to microtubules (*ndc80-1).* For this purpose we combined *alk1*Δ*alk2*Δ with *mif2-3* or with *ndc80-1*. At non permissive temperature (37°C) both mutations are lethal, while at semipermissive temperatures (36°C for *mif2-3* and 28°C for *ndc80-1*) the mutants grow almost normally, but show defects in spindle attachment or elongation, and activate the SAC (Gardner et al., 2001 and Figure 6C). Figure 6B shows that in the absence of haspin both mutants lose viability even at semipermissive temperature. In these cells the SAC is fully functional, as shown by the stabilization of Pds1 (Figure 6C). This indicates that haspin function is essential to allow cells that activate the SAC to properly complete mitosis and survive.

**Discussion**

Haspin is an atypical protein kinase that has been conserved throughout eukaryotic evolution; it is also present in the *Encephalitozoon cunicoli* genome, which contains just 2000 genes and is only ~2.9 Mb long (Katinka et al., 2001). This extreme conservation suggests a critical function for haspin. In human, *A. thaliana* and fission yeast cells, haspin has been reported to be responsible for the phosphorylation of H3Thr3. This phosphosite is a docking site for the chromosome passenger complex (CPC), including Aurora B kinase, that is critical for proper chromosomal alignment at the metaphase plate and for chromosomal movement (Kelly et al., 2010; Wang et al., 2010; Yamagishi et al., 2010; Ashtiyani et al., 2011). *S. cerevisiae* contains two haspin paralogs, coded by *ALK1* and *ALK2,* but H3Thr3 does not seem to be phosphorylated. This suggests that budding yeast may be a good model to identify other processes where haspin kinase is involved. We report that yeast cells lacking *ALK1* and *ALK2* are exquisitely sensitive to microtubule depolymerizing drugs (i.e. benomyl, nocodazole). Recent work suggested that chemical inhibition of haspin may impair the activation of SAC (De Antoni et al., 2012; Wang et al., 2012); however, haspin downregulation via siRNA does not seem to produce the same SAC defect (Wang et al., 2012). By monitoring the stabilization of the securin Pds1 and the separation of sister chromatids, prevented by the SAC, after exposure to nocodazole or with conditional mutations affecting kinetochore functionality, we show that yeast cells completely lacking haspin properly activate the SAC. This excludes that a SAC defect could be explain the nocodazole-induced cell death. After the release from a nocodazole treatment, the kinetics of reassembly of the mitotic spindle is normal in *alk1*Δ*alk2*Δ cells, but surprisingly mutant cells exhibit a highly penetrant spindle positioning defect. Indeed, after a transient nocodazole treatment, cells lacking haspin position the mitotic spindle entirely within the bud, where it elongates and drives nuclear division, producing an anucleated mother and a binucleated

daughter cell. To our knowledge, while nuclear mispositioning in the bud has been reported and linked to a FEAR defect (Ross and Cohen-Fix, 2004), this is the first observation reporting exit from mitosis where spindle elongation and nuclear division are restricted within the bud. The SPOC has been shown to be triggered when the mispositioned spindle is within the mother cell (Fraschini et al., 2008; Bertazzi et al., 2011); in our mutant, the SPOC is functional (data not shown), but it is not activated likely because the spindle is mispositioned within the bud. All these data suggest that haspin loss does not interfere with microtubule dynamics, and it does not affect the Kar9 and Dyn1 pathways; instead, haspin function becomes essential after transient mitotic arrest. Indeed, all the phenotypes described above can be observed by genetically inducing a mitotic delay in *alk1*Δ*alk2*Δ cells. Our results indicate that yeast haspin has a previously undescribed role in allowing cells to tolerate the mitotic delay induced by SAC activation and re-establish the proper spindle positioning. Spindle positioning is governed by the actin cytoskeleton. After a transient metaphase arrest, cells lacking haspin exhibit a misdistribution of actin, which accumulates within the bud. Actin dynamics is modulated by an intricate network including septins, polarisome complex and formins. We show that, similarly to what happens with actin, loss of haspin causes the accumulation of formins and polarisome components in the bud. The mechanism controlling the recruitment of these proteins to their proper location is still largely unknown, making it difficult to determine the direct target of haspin kinase. Preliminary results suggest that the phosphorylation status of Bud6 and Bnr1 is not influenced by loss of haspin (not shown). High-throughput screenings for kinase substrates and for physical and genetic interactions, implicated several polarization proteins with Alk1 and Alk2 (Bodenmiller et al., 2010; Breitkreutz et al., 2010; Fiedler et al., 2009; Sharifpoor et al., 2012); further studies will be required to identify other players involved in the establishment of cell polarity that could be targeted by haspin. Polarity factors have to redistribute during mitosis (Geymonat et al., 2009), and haspin-defective cells show a clear defect

in such redistribution, suggesting that haspin plays an important function in maintaining the coupling between cell cycle progression and redistribution of cell polarity factors that are crucial for correct cell division after cell cycle restart. Failure to properly localize polarity cues causes a prolonged hyperpolarization during a mitotic arrest, which sustain the forces pulling the spindle toward the daughter cell. The absence of Bud6 from the bud neck and the restriction of actin and polarisome within the daughter affect the establishment of a balancing force pulling toward the mother, resulting in mispositioning of the mitotic spindle. A similar unbalance of these forces has been suggested to explain the "daughterly" nuclear positioning observed in the absence of sister chromatid separation in *esp1-1* mutants, during an unperturbed cell cycle (Ross and Cohen-Fix, 2004). Intriguingly, we found that expression of *A. thaliana* haspin in *alk1*Δ*alk2*Δ cells suppresses the benomyl sensitive phenotype and partially rescues the nuclear segregation defect (Figure S5), strongly suggesting that haspin function in cell polarity is conserved throughout evolution. Asymmetric cell division is a fundamental process for development and tissue homeostasis in a variety of organisms, and requires the establishment of a proper cell polarization program. During embryogenesis, asymmetric divisions are responsible for the generation of a diverse array of different cell types. In adult organisms, regulation of asymmetrical division in stem cells is required to balance self-renewal and commitment to differentiation, contributing to tissue homeostasis. Thus, the implications of a defective regulation of asymmetric cell division are extremely relevant: yeast cells fail to produce a vital colony, embryonic development may be abnormal, and individuals could develop cancer or suffer tissue degeneration. Given the fundamental function that haspin has in modulating cell polarity in budding yeast, it will be interesting to investigate whether a similar role is shared also in mammalian cells and loss of haspin activity affects the correct execution of asymmetric cell division.

**Acknowledgements**

We thank L. Diani, the CIMA facility at the University of Milano, Virginie Georget (MRI microscopy facility, CRBM, Montpellier) for technical assistance, Alessio Bolognesi and Yves Barral (ETH, Zurich) for helping in the analysis of septin dynamics. M. Peter, M. Weinreich, M. Segal and D. Pellman are acknowledged for donating plasmids and strains.

This work was supported by grants from AIRC (M.M-F), Fondazione Cariplo, MIUR (M.M-F, and P.P.) and Agence Nationale de la Recherche [ANR-09-BLAN-0125-01] (S.P.)

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Part II

**Legend to figures**

**Figure 1. Yeast Haspin deletions cause sensitivity to microtubules-depolymerizing agents.**

**A**. Serial dilutions of the indicated yeast strains grown on YPD (mock) or YPD + 8 µg/ml benomyl plates. **B**. Wild-type, *alk1Δalk2Δ* and kinase-deficient double mutant (*alk1kdalk2kd*) treated as in **A**. **C**. Time-course of Pds1 stability in cells arrested in α-factor and released in 20 µg/ml nocodazolecontaining medium. * indicates a protein cross-reacting with the anti-HA Ab, which can be used as a loading control. **D**. Analysis of sister chromatid separation in cells where chromosome V was labeled with GFP 35 kb away from the centromere, treated as in **C**. The inset presents examples of the fluorescence images showing normal sister chromatid cohesion (wild-type, *alk1*Δ*alk2*Δ) or premature separation (*mad2*Δ).

**Figure 2. Lack of Haspin impairs mitotic spindle positioning after mitotic arrest.**

**A**. Representative immunofluorescence pictures, showing DNA (DAPI) and the mitotic spindle (Tub), taken at the indicated time-points after nocodazole wash-out. **B**. Quantification of binucleated cells exhibiting incorrect mitosis after treatment as in **A**. **C**. Kinetic analysis of spindle orientation after release from nocodazole. Selected photograms from a time-lapse experiment (Supplementary Movies 1, 2) using strains expressing Tubulin-GFP. The time-point of each photogram is indicated. **D**. Cells were treated with 10µg/ml α-Factor, then released in medium containing 10µg/ml of nocodazole to induce mitotic arrest. Nocodazole was then washed out and samples were collected after 60 min in YPD. Nuclei were stained with DAPI and quantified as indicated. **E**. Representative images of Concanavalin A stained wild-type and *alk1*Δ*alk2*Δ cells carrying Tub1-GFP, after nocodazole wash-out **F**. Wild-type and *alk1*Δ*alk2*Δ cells, expressing *GAL1-CDC20*, were grown overnight in medium

containing raffinose and galactose, and pre-synchronized in G1 with α-factor 5 µg/ml. When 95% of the cells were unbudded, the cultures were washed and resuspended in medium containing glucose for 3,5 hours. Metaphase arrested cells were then released in medium containing raffinose and galactose to allow *CDC20* expression. Samples were collected 60 min after nocodazole release in galactose and immunofluorescence was performed. Selected images are shown. **G** Quantification of the experiment in **F**.

**Figure 3. Haspin is essential for proper organization of cell polarity factors in mitosis.**

**A**. Actin distribution in the indicated strains was analyzed by fluorescent microscopy after phalloidin staining. Samples were collected at the indicated time points after nocodazole wash-out; selected images are shown. with quantification of at least 200 cells/sample. **B**. Concanavalin A stained cells were analysed for actin distribution as in **A**. Selected images are presented. **C**. Overnight cultures of wild-type, *cdc12-6, alk1Δalk2Δ, alk2Δalk2Δcdc12 -6* grown at permissive temperature were treated with nocodazole 10µg/ml for 2,5 hours, shifted to non-permissive temperature for 45 min, and then released in fresh medium at the non-permissive temperature. Samples were collected at 20 min and stained with phalloidin. Quantification of at least 200 cells/samples were shown. **D**. Cells were treated as in C but performing immunofluorescence against tubulin and DAPI staining. Selected images are shown with quantification of binucleated cells exhibiting incorrect mitosis

**Figure 4. In *alk1Δalk2Δ* cells Bnr1 is mislocalized and the spindle pulling forces are unbalanced.**

**A**. GFP-Bnr1 localization was analyzed by fluorescence microscopy. Samples were collected at the indicated time-points after 2,5 hours in nocodazole; representative images are shown. The fluorescence intensity along the mother-daughter cell axis (rectangular area) has been quantified and is reported in

arbitrary units in the line graphs. The locations corresponding to the bud neck and the bud tip are marked accordingly. The fraction of the total fluorescence that is localized at the bud neck and bud tip is reported as a bar graph. **B**. Quantification of the different categories observed in the experiment described in **A**. At least 200 cells/sample were scored. **C**. Serial dilution of exponentially growing cultures were plated on benomyl (8 µg/ml) and incubated at 23°C for 3 days. **D**. Quantification of binucleated cells exhibiting incorrect mitosis 60 min after the release from a nocodazole treatment of the indicated yeast strains.

**Figure 5. Localization of GFP-Bud6 in absence of haspin.**

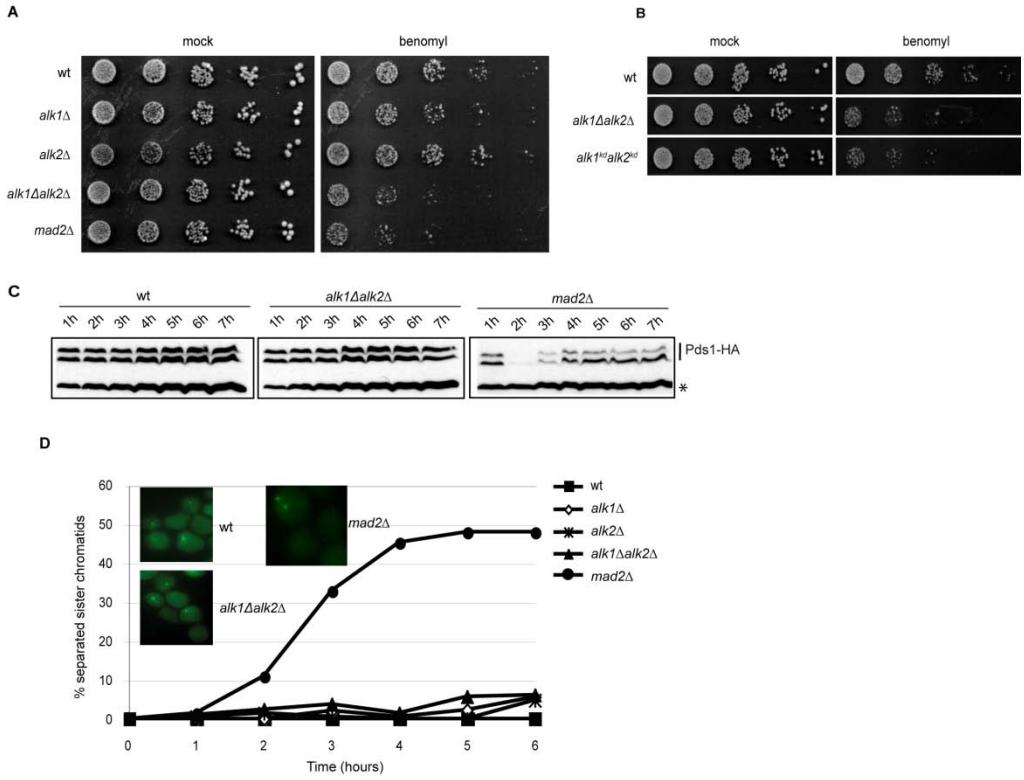
**A**. GFP-Bud6 localization was analysed by fluorescence microscopy at various time-points after the release from a nocodazole treatment. Representative images are shown. The fluorescence intensity along the mother-daughter cell axis (rectangular area) has been quantified and is reported in arbitrary units in the line graphs. The locations corresponding to the bud neck is marked accordingly. **B**. Quantification of the experiment in **A**.

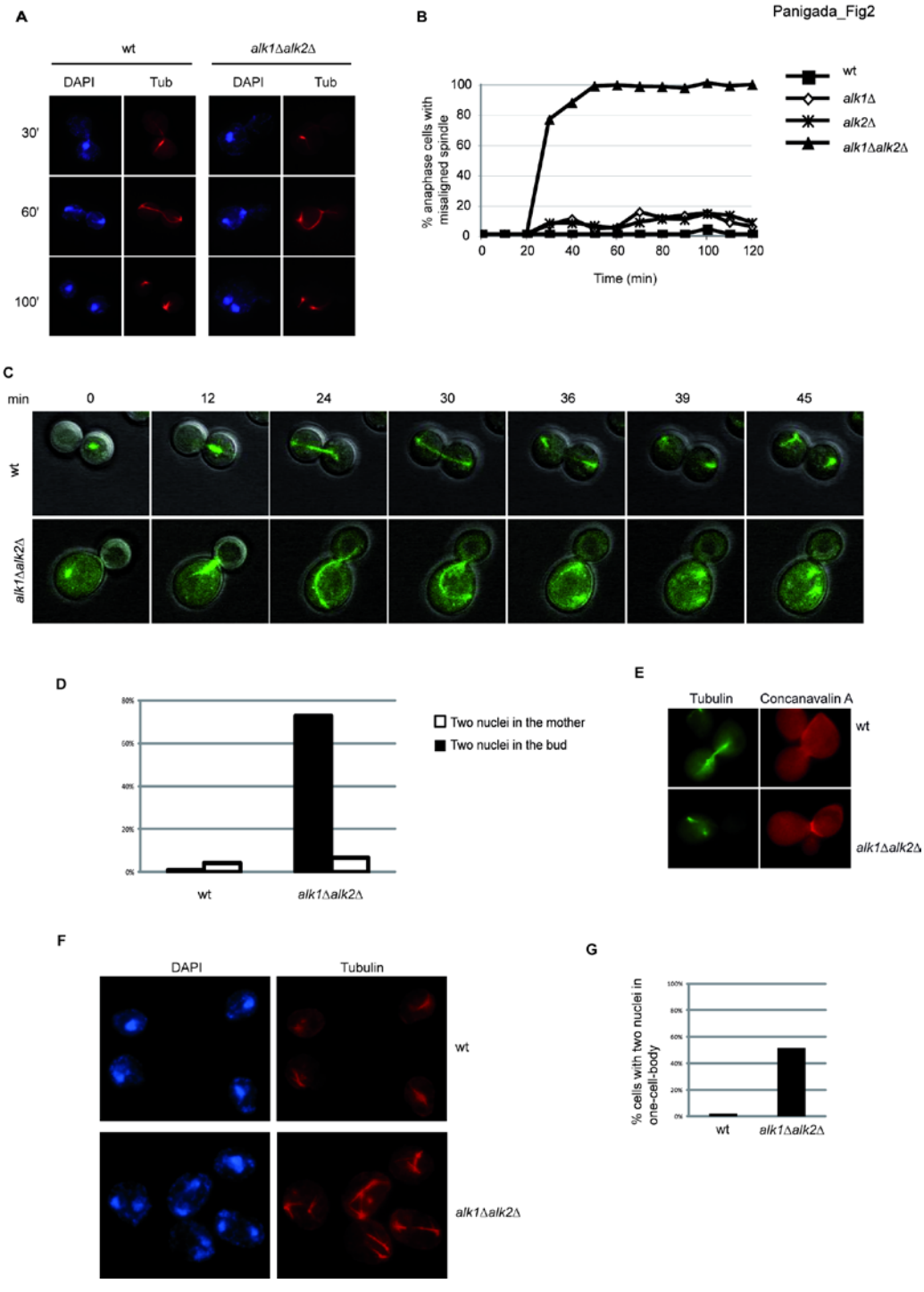
**Figure 6. Haspin function is required for proper localization of polarity cues during an unperturbed cells cycle and is essential to tolerate a SAC-dependent mitotic arrest**

**A** GFP-Bud6 localization in synchronized cultures was analysed by fluorescence microscopy at the indicated time-points after the release from α-factor, selected images are shown. Quantification of the experiments is shown in the histogram. At least 200 cells/sample were scored. **B**. Serial dilution of exponentially growing cultures of the indicated yeast strains were plated on YPD and incubated for 2 days at the indicated temperatures. **C**. Time course analysis of Pds1-HA stability. Cells of the indicated strains were arrested in G1 at 25°C, and released in YPD at 37°C. Samples were collected at the indicated time points and Pds1 levels were analysed by western blotting with 12CA5 (HA) antibodies.

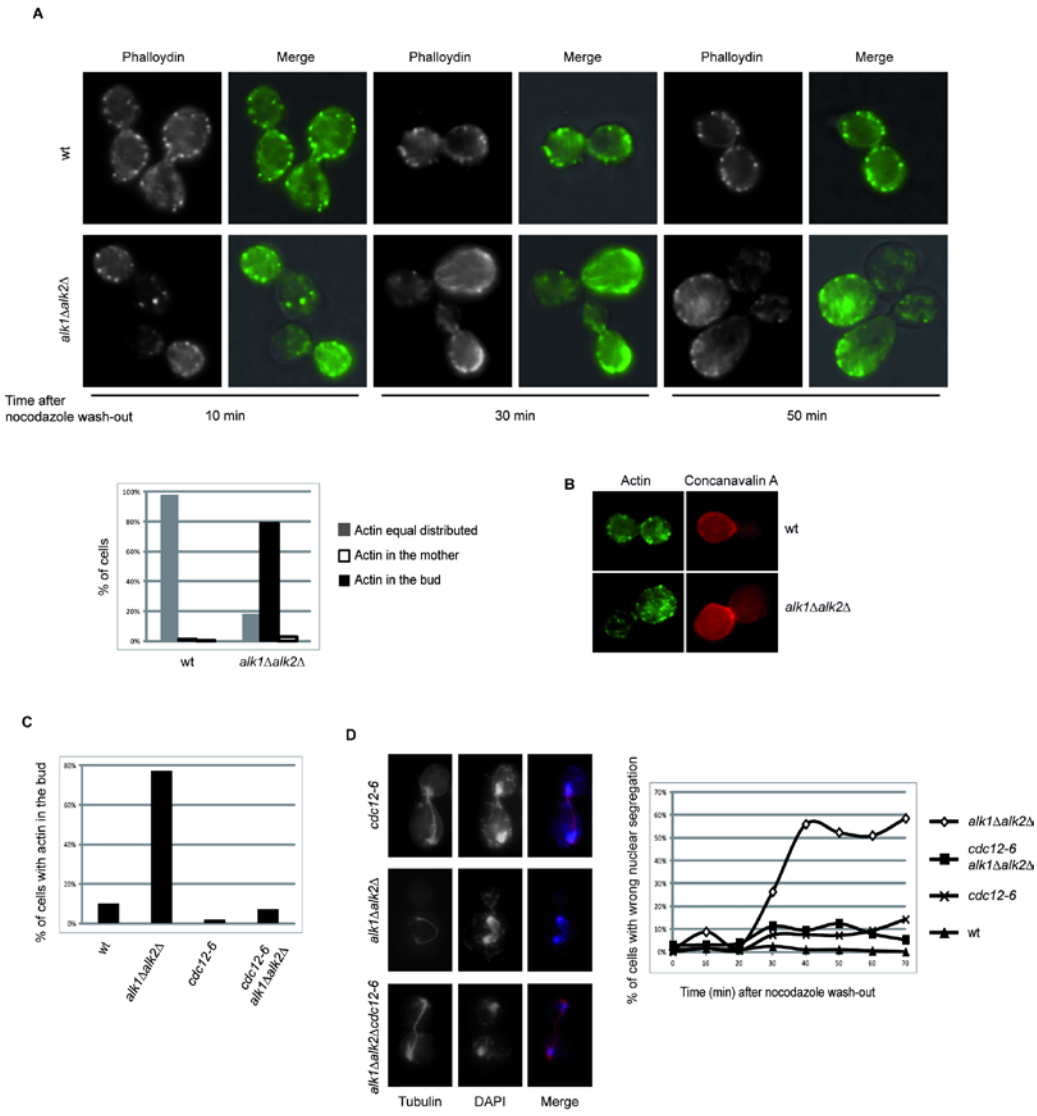
Part II

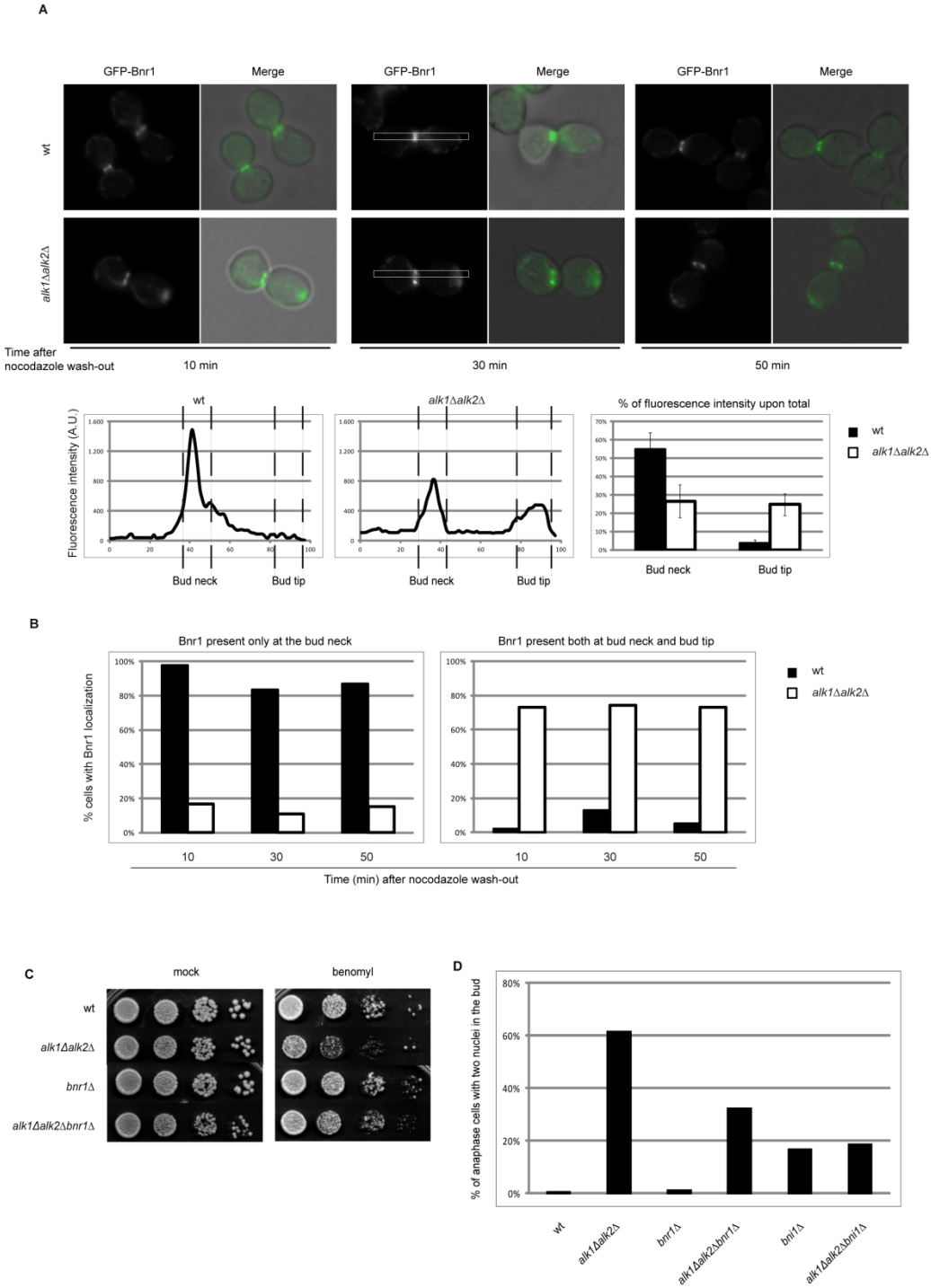
Panigada_Fig1



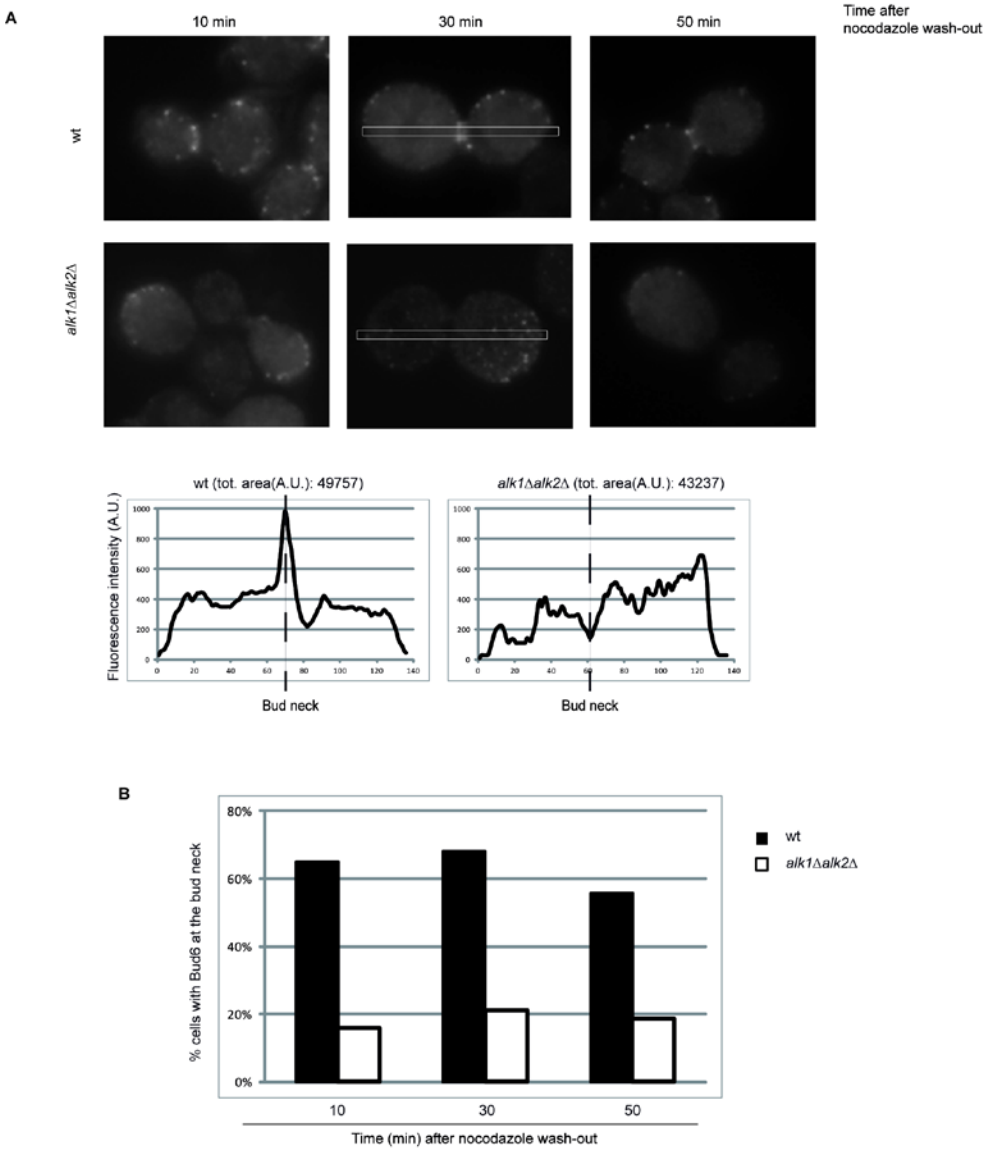

A
wt
alk1Δalk2Δ
DAPI
Tub
DAPI
Tub
30'
60'
100'
B
Panigada_Fig2
% anaphase cells with misaligned spindle
100
80
60
40
20
0
0
20
40
60
80
100
120
Time (min)
wt
alk1Δ
alk2Δ
alk1Δalk2Δ
C
min
0
12
24
30
36
39
45
wt
alk1Δalk2Δ
D
80%
60%
40%
20%
0%
wt
alk1Δalk2Δ
Two nuclei in the mother
Two nuclei in the bud
E
Tubulin
Concanavalin A
wt
alk1Δalk2Δ
F
DAPI
Tubulin
wt
alk1Δalk2Δ
G
% cells with two nuclei in one-cell-body
100%
80%
60%
40%
20%
0%
wt
alk1Δalk2Δ

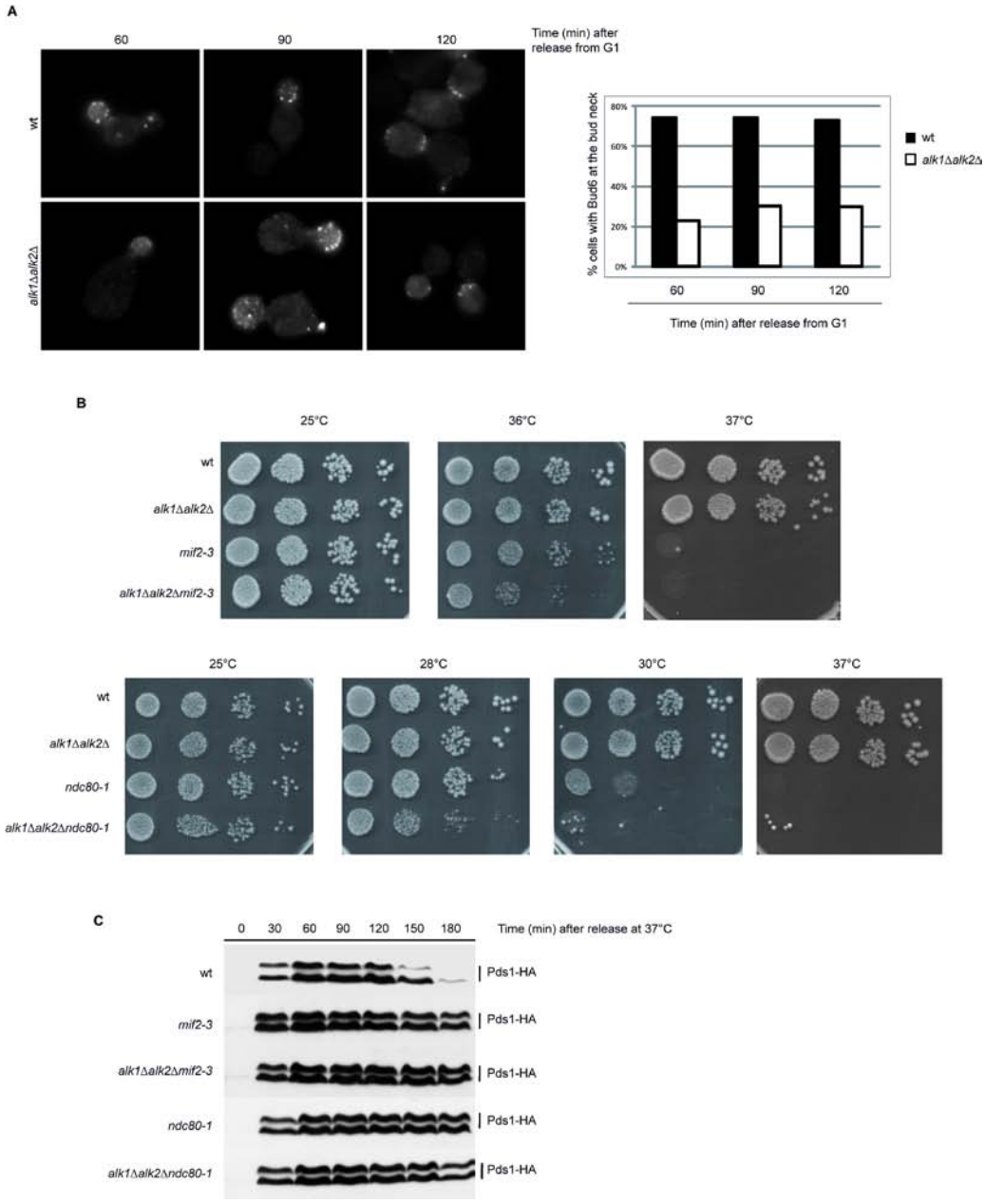
Panigada_Fig3




A
GFP-Bnr1
Merge
wt
alk1Δalk2Δ
Time after nocodazole wash-out
10 min
30 min
50 min
Fluorescence intensity (A.U.)
Bud neck
Bud tip
% of fluorescence intensity upon total
B
Bnr1 present only at the bud neck
Bnr1 present both at bud neck and bud tip
% cells with Bnr1 localization
Time (min) after nocodazole wash-out
C
mock
benomyl
bnr1Δ
alk1Δalk2Δbnr1Δ
D
% of anaphase cells with two nuclei in the bud

Panigada_Fig5




A
60
90
120
Time (min) after release from G1
wt
alk1Δalk2Δ
% cells with Bud6 at the bud neck
80%
60%
40%
20%
0%
60
90
120
Time (min) after release from G1
wt
alk1Δalk2Δ
B
25°C
36°C
37°C
wt
alk1Δalk2Δ
mif2-3
alk1Δalk2Δmif2-3
25°C
28°C
30°C
37°C
wt
alk1Δalk2Δ
ndc80-1
alk1Δalk2Δndc80-1
C
0 30 60 90 120 150 180
Time (min) after release at 37°C
wt
mif2-3
alk1Δalk2Δmif2-3
ndc80-1
alk1Δalk2Δndc80-1
Pds1-HA
Pds1-HA
Pds1-HA
Pds1-HA
Pds1-HA

Inventory of supplemental materials

Figure S1

The results presented in the figure S1 show that after transient nocodazole arrest haspin deleted yeast cells accumulates binuclated cells that are not alive, and that this correlates with the kinase activity of the protein, confirming that the lethal event is not a failure in the activation of the SAC, as demonstrated in figure 1.

Figure S2

In figure 2, we show that *alk1*Δ*alk2*Δ cells exhibit defects in spindle reorientation. In figure S2 we exclude genetic interactions between Alk1, Alk2 and the well established pathways of spindle positioning in yeast, whose main actors are Dyn1 and Kar9.

Figure S3

Figure 3 shows that haspin deleted yeast cells have an impaired distribution of actin after mitotic arrest. This phenotype is fully rescued by disruption of the septin complex, leading to the possibility that haspin mutant may be involved in the regulation of the dynamics of this complex. The results in figure S3 show that there is no different in the status of the septin in wt and in the haspin mutan, analyzing them by FRAP and by checking the phosphorylation status of Shs1, a member of the complex, whose phosphorylation correlates with changing in the septin ring dynamics.

Figure S4

The figure shows that the localization of the polarisome complex member Spa2 is impaired in the haspin deleted cells reinforcing the idea that different member of the polarity cap are mislocalized. In fact also the localization of Bud6, another member of the polarisome, is impaired as presented in the figure 5.

Figure S5

The figure shows that the lethality of haspin deleted cells on benomyl and the nuclear segregation defect observed in figure 1, can be rescued by expression of Arabidopsis thaliana haspin, confirming the evolutionary conservation of the protein.

Movie S1 and Movie S2

The movies show the kinetics of spindle rebuilding and disassembly after nocodazole treatment, in which is clear that the lethality of alk1Δalk2Δ cells is not caused by problem in the organization of the mitotic spindle but in its alignment.

**Supplemental figure legends.**

**Figure S1. Lack of yeast haspin leads to cell death after nocodazole treatment.**

**A**. Quantification of cell survival was obtained by plating (300 cells per plate) wild-type and *alk1Δalk2Δ* cells, after treatment with or without nocodazole 15µg/ml for 6 hours. Colonies were counted after 2 days of incubation. The graph is representative of three independent experiments. Error bars describe standard deviation. Cells from the same samples were also plated on YPD in the presence of Phloxine B which stains in red the colonies containing dead cells. **B**. Quantification of the reformation of bipolar spindles in wild-type and *alk1*Δ*alk2*Δ cells after nocodazole release. Cells were treated with nocodazole for 2.5 hours and, after wash-out, samples were collected at different time-points and tubulin was detected by immunofluorescence. **C**. Quantification of binucleated cells with incorrect anaphase spindle after release from nocodazole treatment of the kinase-defective haspin mutant strains. **D**. Cytofluorimetric analysis of Sytox green stained cells pre-synchronized in G1, arrested in nocodazole 10 µg/ml and released into fresh medium without the drug. 1C and 2C indicate the pre-replicative (G1) and post-replicative (G2-M) DNA contents; the arrow indicates the re-replicating population.

**Figure S2. Haspin deletion does not affect spindle reassembly and spindle positioning pathways.**

**A**. Serial dilutions of exponentially growing cultures of the indicated strains were plated at different temperature. Plates were scored after two days. **B** Cells of the indicate strains were treated with nocodazole for 2,5 hours; samples were collected 60 min after removal of the drug, and stained with DAPI. The histogram reports the quantification of cells undergoing anaphase in the bud.

**Figure S3. Septin ring dynamics is not impaired in haspin-deleted cells.**

**A**. Shs1 phosphorylation in cells released from a nocodazole arrest was analysed by western blotting. **B**. Septin ring dynamics was estimated by FRAP analysis using strains expressing GFP-Cdc12. Half of the ring was bleached at time 0. Fluorescence recovery was measured over time and plotted as fraction of fluorescence intensity relative to time 0 (unbleached). Results from 5 independent experiments are presented, and a representative cell is shown. The white rectangle indicates the bleached area.

**Figure S4. Localization of the Spa2 polarisome component after nocodazole wash-out.**

**A**. wild-type and *alk1*Δ*alk2*Δ cells, expressing Spa2-GFP, were arrested in nocodazole and released in fresh medium. Samples were taken at different time-points after the release and fixed in ethanol. Representative pictures are shown. The fluorescence intensity along the mother-daughter cell axis (rectangular area) has been quantified and is reported in arbitrary units in the line graphs. **B**. Time course analysis of the distribution of Spa2-GFP from the experiment in **A**. At least 200 cells/samples were scored.

**Figure S5. At-haspin suppresses the benomyl sensitivity of yeast *alk1*Δ*alk2*Δ cells.**

**A**. Serial dilutions of the indicated yeast strains grown on YP Gal/Raf (mock) or YP Gal/Raf + 8 µg/ml benomyl plates. **B**. wild-type or *alk1*Δ*alk2*Δ cells, carrying a plasmid expressing *AtHaspin* or an empty vector, were arrested in nocodazole for 2,5 hours and released in YP Gal/Raf. Samples were collected after 80 min and stained with DAPI. The histogram reports the frequency of cells undergoing through anaphase with both nuclei in the bud.

**Supplemental movie legends**

**Supplemental movie 1**. Spindle reassembly in wild-type cells after nocodazole block and release. Time lapse microscopy of cells carrying GFP-Tub1 (green) merged with DIC images. Each time-point was taken 1 min after the previous one. These cells are the same shown in Figure 2C.

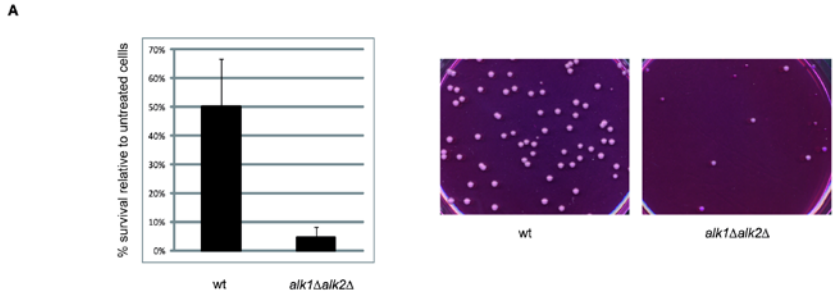
**Supplemental movie 2**. Spindle reassembly in *alk1*Δ*alk2*Δ cells after nocodazole block and release. Time lapse microscopy of cells carrying GFP-Tub1 (green) merged with DIC images. Each time-point was taken 1 min after the previous one. These cells are the same shown in Figure 2C

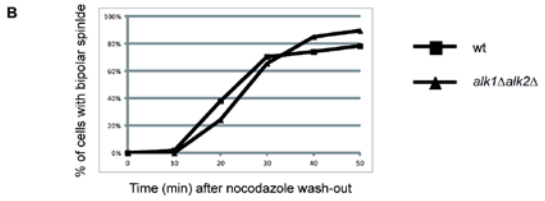
**Table S1. Yeast Strains used in this study**

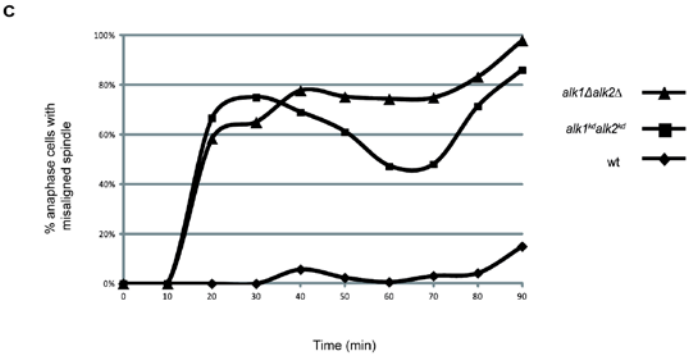
| Name | Relevant genotype | Source |
|---|---|---|
| K699 | *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11, 15 ura3 MATa* | Kim Nasmyth |
| 601 | *leu2*::LEU2 tetR-GFP *ura3*::URA3 224Xtet O *MATa* | S.Piatti |
| 1061 | *mad2Δ::TRP MATa* | S.Piatti |
| 1733 | Pds1-HA:LEU *MATa* | S.Piatti |
| 1791 | GFP-Tub1:HIS3 *MATa* | S.Piatti |
| yAN23 | *alk2Δ::HIS MATa* | This work |
| yAN42 | *alk1Δ::kan MATa* | This work |
| yAN43 | *alk1Δ::kan alk2Δ::HIS MATa* | This work |
| yAN257 | *alk2Δ::HIS Leu2*::LEU2 tetR-GFP *ura3*::URA3 224XtetO *MATa* | This work |
| yAN270 | *alk1Δ::kan Leu2*::LEU2 tetR-GFP *ura3*::URA3 224Xtet O *MATa* | This work |
| yAN271 | *alk1Δ::kan alk2Δ::HIS Leu2*::LEU2 tetR-GFP *ura3*::URA3 224XtetO *MATa* | This work |
| yAN272 | *mad2Δ::TRP Leu2*::LEU2 tetR-GFP *ura3*::URA3 224Xtet O *MATa* | This work |
| yAN308 | Alk1$^{kd}$:URA3 Alk2$^{kd}$:URA3 *MATa* | This work |
| yAN336 | *alk1Δ::nat alk2Δ::HIS MATa* | This work |
| yPG12 | Pds1-HA:LEU *alk1Δ::kan Δalk2::HIS MATa* | This work |
| yPG13 | Pds1-HA:LEU *mad2Δ::TRP MATa* | This work |
| yPG252 | *alk1Δ::nat alk2Δ::kan* GFP-Tub1:HIS3 *MATa* | This work |
| yPD80 | *GAL-CDC20-TRP MATa* | This work |
| yPD81 | *GAL-CDC20-TRP alk1Δ::nat alk2Δ::HIS MATa* | This work |
| yPD85 | *[GFP-BUD6-URA3] MATa* | This work |
| yPD86 | *alk1Δ::nat alk2Δ::HIS [GFP-BUD6-URA3] MATa* | This work |
| yPG386 | *SPA2-GFP-URA3 MATa* | This work |
| yPG408 | *alk1Δ::kan alk2Δ::HIS SPA2-GFP-URA3 MATa* | This work |
| 5183 | *cdc12-6 MATa* | S. Piatti |
| yAN348 | *cdc12-6 alk1Δ::kan alk2Δ::HIS MATa* | This work |
| yPD118 | *[GFP-BNR1-URA3] MATa* | This work |
| yPD117 | *alk1Δ::nat alk2Δ::HIS [GFP-BNR1-URA3] MATa* | This work |
| yPG322 | *alk1Δ::kan alk2Δ::kan MATa* | This work |
| yPD111 | *bnr1Δ::kan MATa* | This work |
| yPD112 | *alk1Δ::nat alk2Δ::HIS bnr1Δ::kan MATa* | This work |
| yAN342 | *bni1Δ::kan MATa* | This work |
| yPD151 | *alk1Δ::nat alk2Δ::HIS bni1Δ::kan MATa* | This work |
| yPD177 | *mif2-3 MATa* | This work |
| yPD178 | *alk1Δ::nat alk2Δ::HIS mif2-3 MATa* | This work |
| 2043 | *ndc80-1 MATa* | This work |
| yPD179 | *alk1Δ::nat alk2Δ::HIS ndc80-1 MATa* | This work |
| yPD199 | *mif2-3* Pds1-HA:LEU *MATa* | This work |

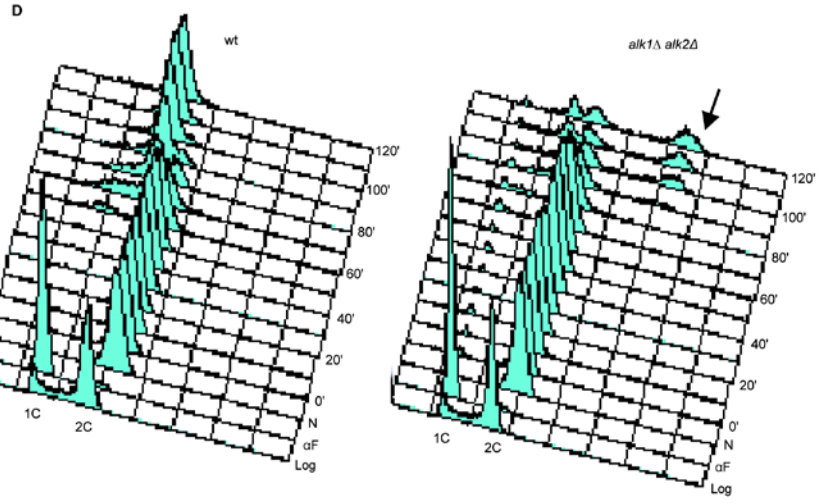
Part II

| | | |
|---|---|---|
| yPD200 | *alk1Δ::nat alk2Δ::HIS mif2-3* Pds1-HA:LEU *MATa* | This work |
| yPD201 | *ndc80-1* Pds1-HA:LEU *MATa* | This work |
| yPD202 | *alk1Δ::nat alk2Δ::HIS ndc80-1* Pds1-HA:LEU *MATa* | This work |

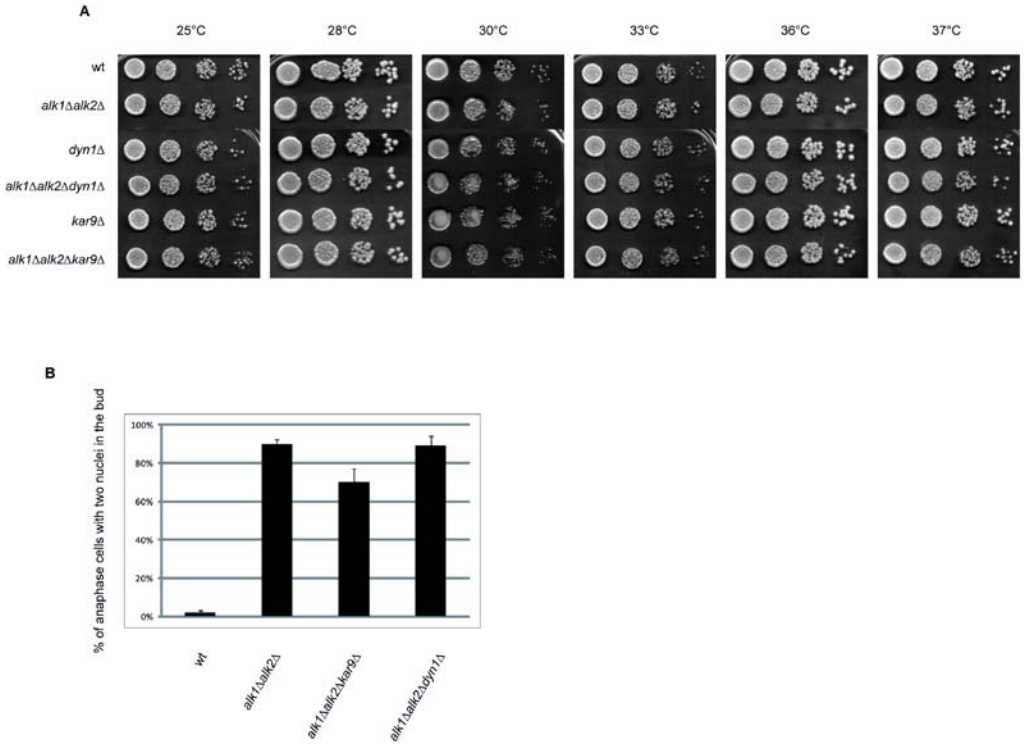

A
% survival relative to untreated cells
70%
60%
50%
40%
30%
20%
10%
0%
wt
alk1Δalk2Δ
wt
alk1Δalk2Δ

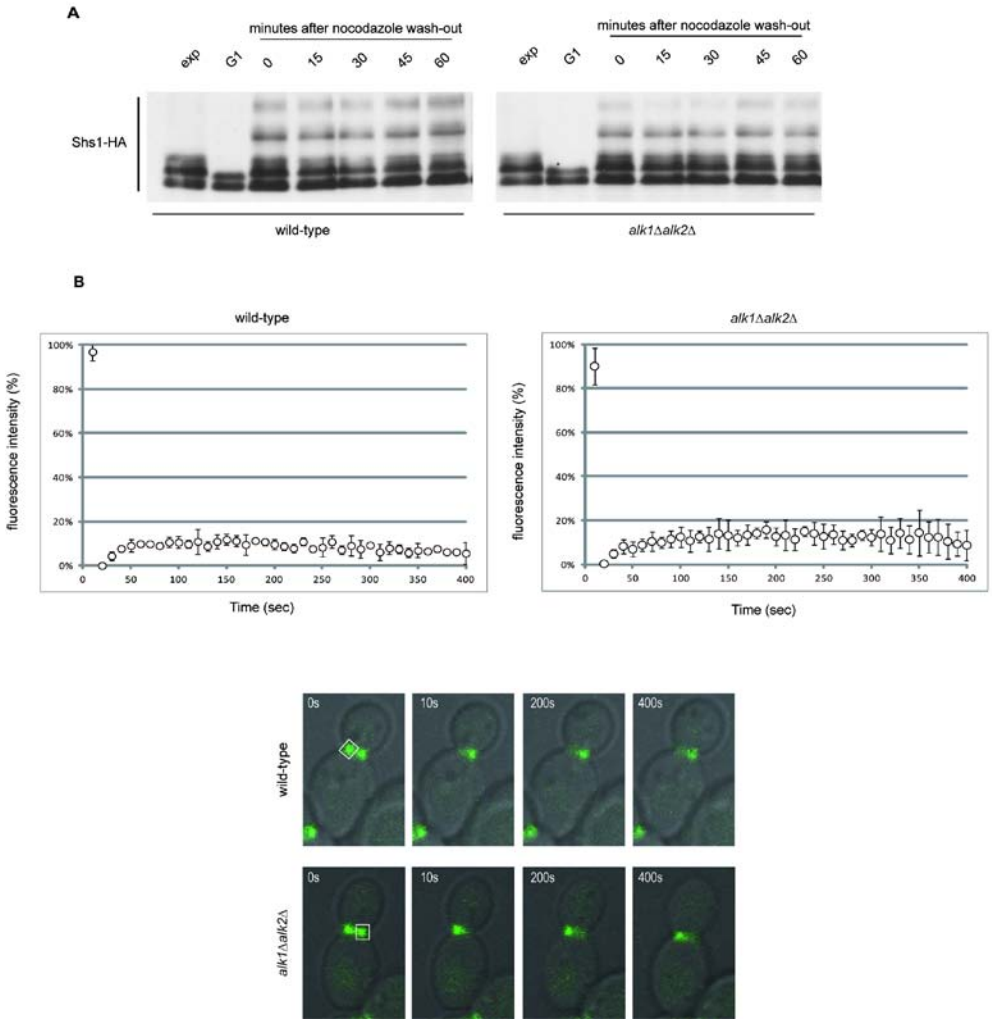

B
% of cells with bipolar spindle
Time (min) after nocodazole wash-out
wt
alk1Δalk2Δ


C
% anaphase cells with misaligned spindle
100%
80%
60%
40%
20%
0%
0
10
20
30
40
50
60
70
80
90
Time (min)
alk1Δalk2Δ
alk1hdalk2hd
wt


D
wt
alk1Δ alk2Δ
120'
100'
80'
60'
40'
20'
0'
N
αF
Log
1C
2C

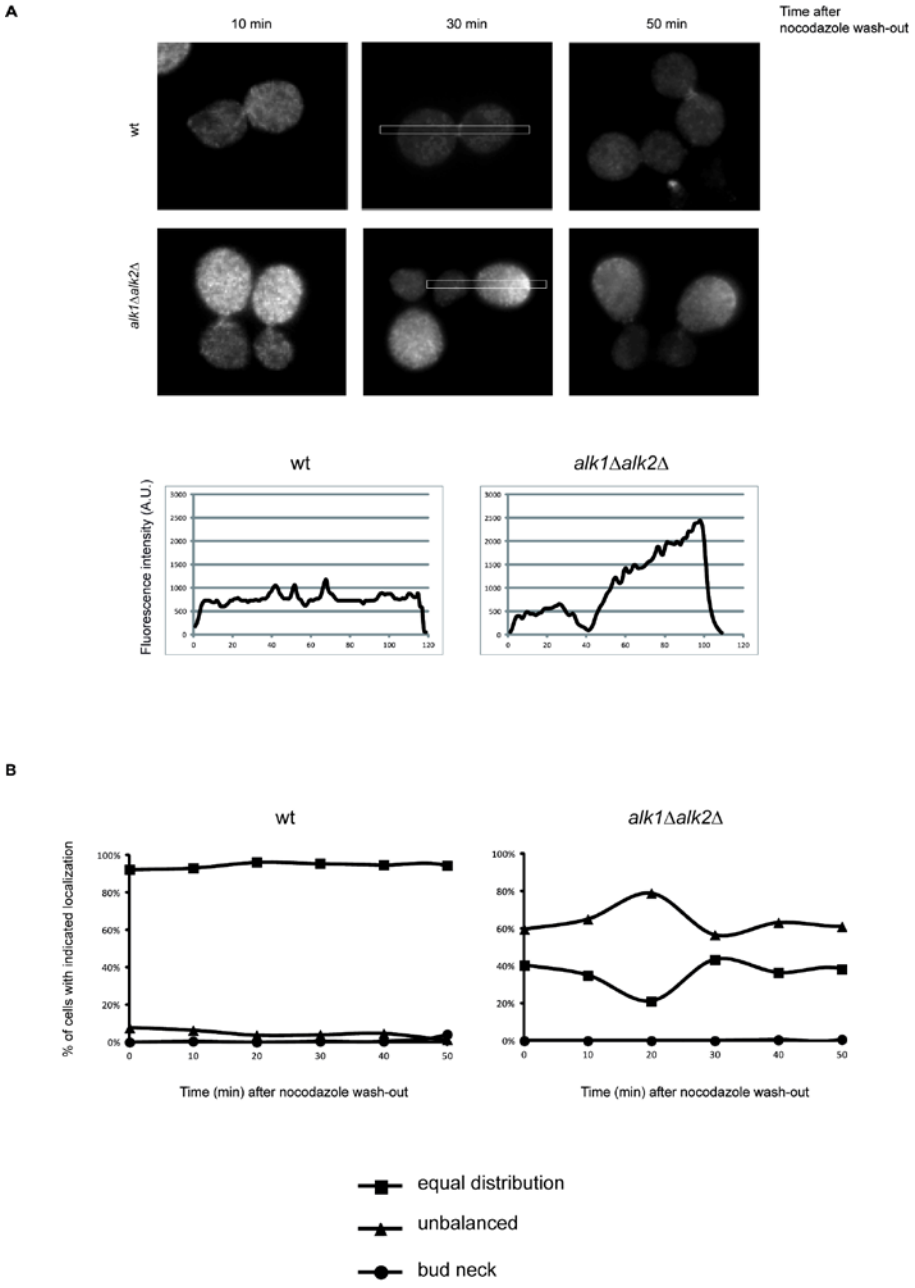
Panigada_Supplemental Fig2

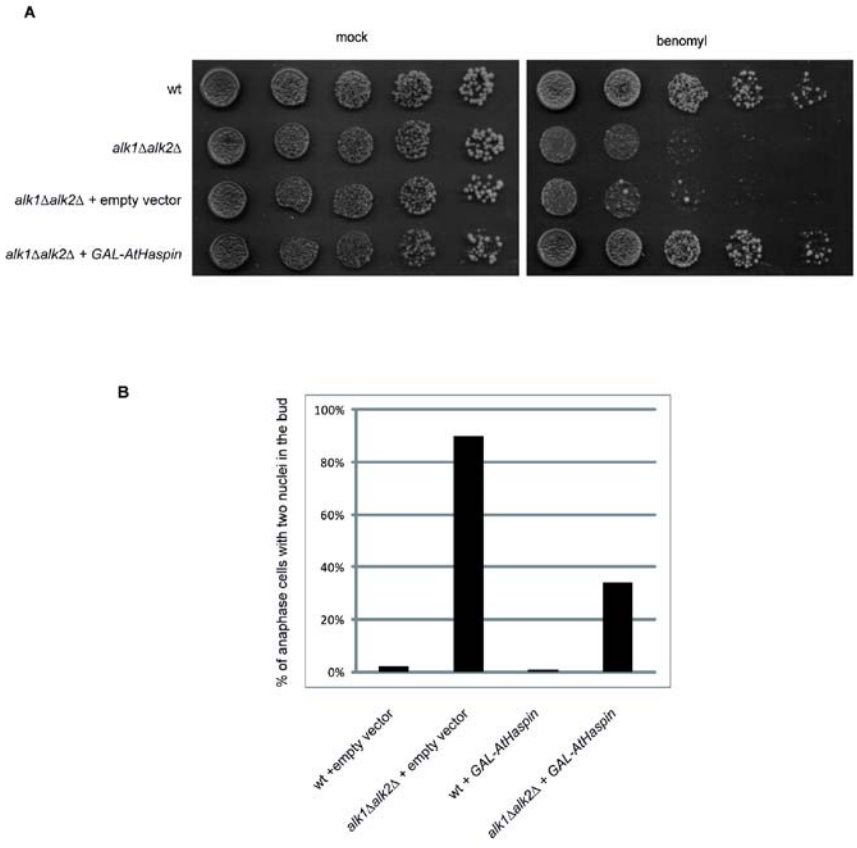



A
minutes after nocodazole wash-out
exp
G1
0
15
30
45
60
Shs1-HA
wild-type
alk1Δalk2Δ
B
wild-type
alk1Δalk2Δ
fluorescence intensity (%)
Time (sec)
0s
10s
200s
400s

Part II

Panigada_Supplemental Fig4




A
mock
benomyl
wt
alk1Δalk2Δ
alk1Δalk2Δ + empty vector
alk1Δalk2Δ + GAL-AtHaspin
B
% of anaphase cells with two nuclei in the bud
100%
80%
60%
40%
20%
0%
wt +empty vector
alk1Δalk2Δ + empty vector
wt + GAL-AtHaspin
alk1Δalk2Δ + GAL-AtHaspin

# Part III

# Supplementary information

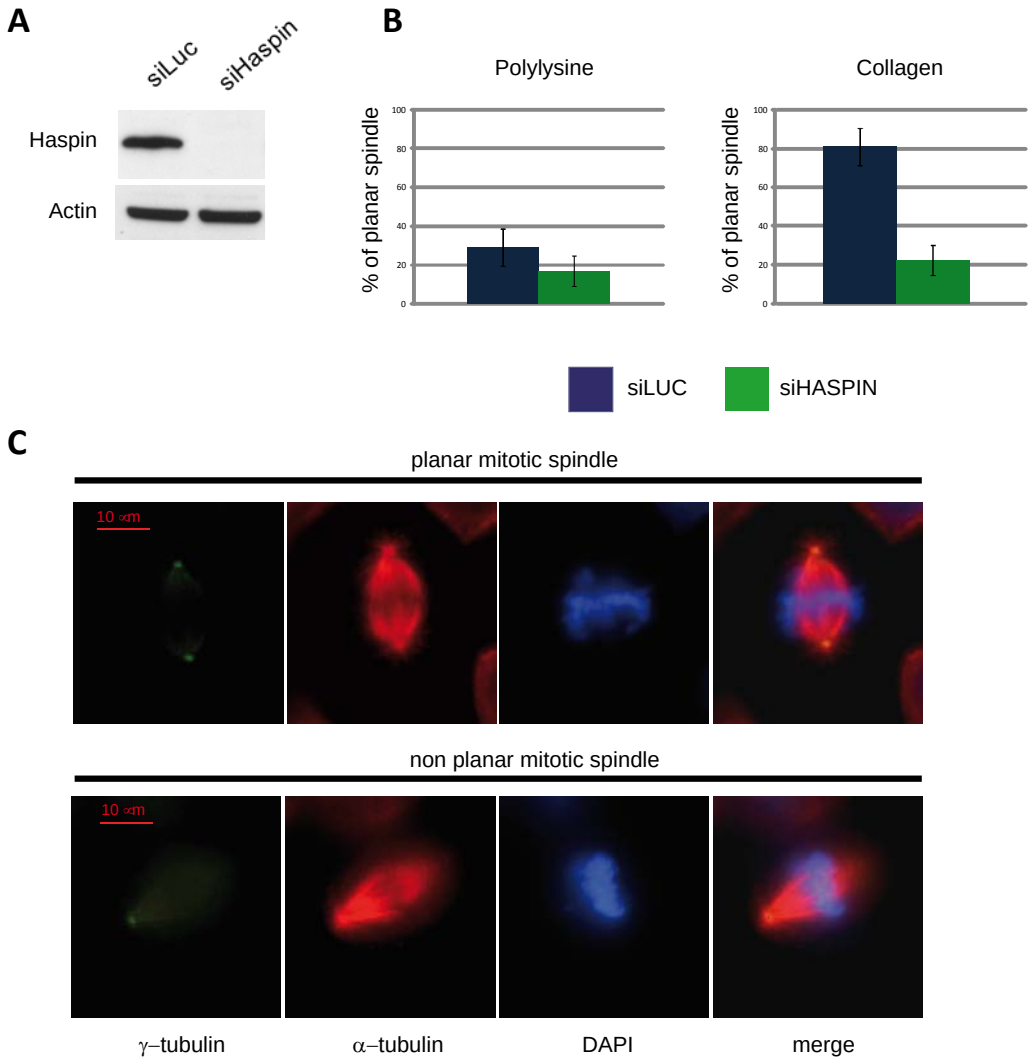



**Fig. S1 Haspin depletion affects spindle orientation in mammalian epithelial cells**
**A.** Western blot analysis to monitor haspin depletion after RNA interference. Antibodies used for western blotting were anti-haspin and anti-actin. **B.** HeLa cells were plated on collagen-coated or polylysine-coated coverslips, synchronized at the G1/S transition with a double thymidine block (DTB) and released; 8 hours after DTB release, mitotic cells were fixed and stained with anti-γ-tubulin antibodies to visualise centrosomes (green) and with anti-α-tubulin antibodies to visualise mitotic spindles (red). At least 100 mitotic cells for each experiment were scored for the presence of a planar spindle and the calculated percentages are represented as histograms. Error bars represent standard deviation derived from at least three independent experiments. **C** A representative picture of planar (upper panels) and non-planar (lower panels) spindles are shown.

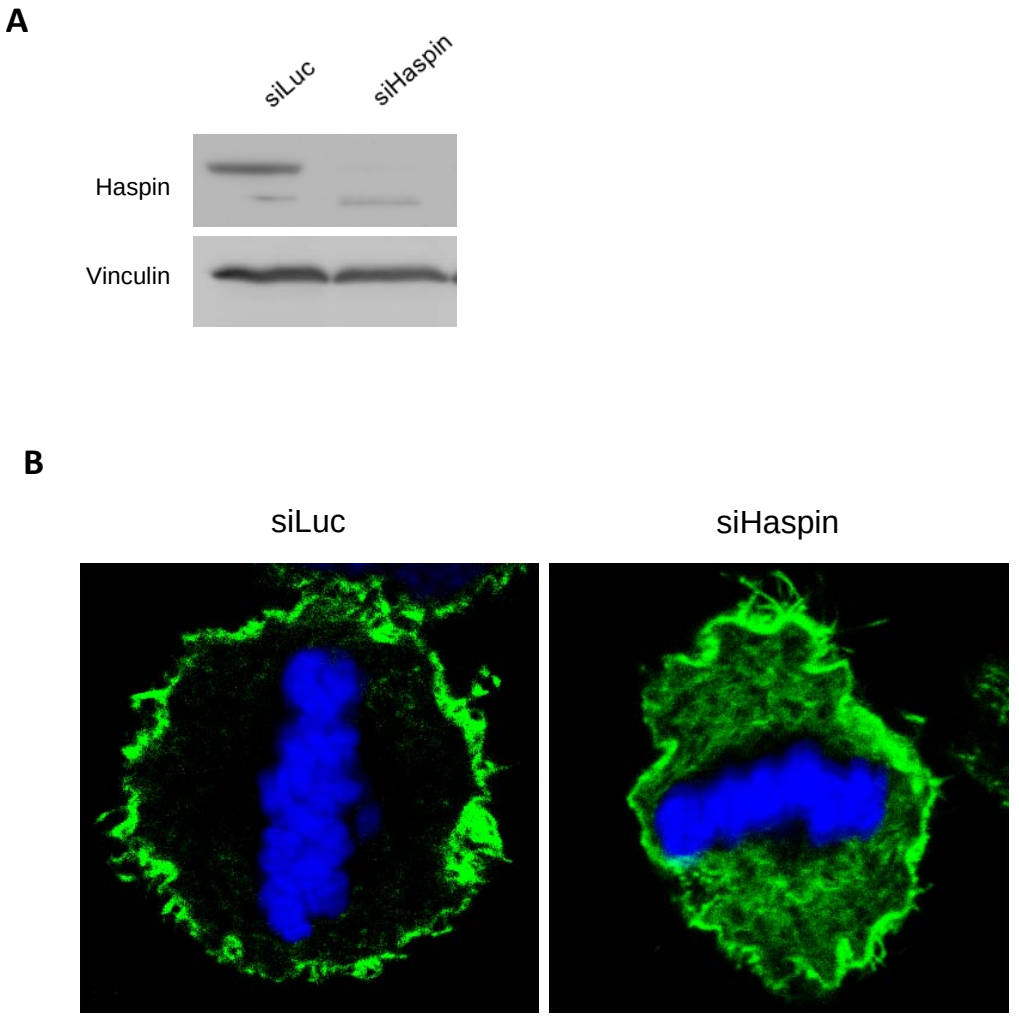



**Fig. S2 Loss of haspin causes disorganization of metaphase actin in HeLa cells**
**A.** Western blot analysis to monitor haspin depletion after RNA interference with antibodies against haspin and vinculin. **B.** HeLa cells were synchronized at the G1/S transition with a double thymidine block (DTB) and released; 8 hours after DTB release, mitotic cells were fixed and stained with Alexa Fluor 488 conjugated phalloydin (actin; green) and DAPI (DNA; blue). Representative pictures are presented.