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HASPIN ROLE IN VESICLE DELIVERY
AND POLARITY DISPERSION

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PART I

ABSTRACT

The atypical protein kinase haspin is conserved in all eukaryotes and promotes the correct alignment of chromosomes on the metaphase plate by recruitment of the CPC. Here, using budding yeast as a model organism, we identified new functions for haspin paralogues (Alk1 and Alk2) in regulating actin and nuclear dynamics. Indeed, we show that haspin mutants experiencing mitotic delays accumulate actin and elongate their spindles entirely in daughter cells, with the consequence of generating anucleated mothers and binucleated daughters that are not vital. These defects are due to a hyperaccumulation of polarity proteins at the bud tip and indeed dispersion of these polarity factors or restoration of their physiological localization reduces the severity of the defects of haspin lacking cells. We also demonstrate that haspin regulates polarisome dispersion by affecting the distribution of Cdc42 activity in cells, particularly regulating the localization of Cdc24, the Cdc42 GEF. We report that localization of this GEF is regulated by Ras in mitosis and that haspin regulates the localization of Ras. We also noticed that loss of haspin causes a polarized delivery of exocytic vesicles towards the bud tip that could explain the defective localization of Ras in *alk1Δalk2Δ* cells.

Moreover, we identified Fab1 kinase as a putative interactor of Alk2 and provide evidences for a interplay between haspin and Fab1 complex.

STATE OF THE ART

All cells derive from pre-existing ones through a precise series of event that allows cell growth and reproduction overall known as the cell cycle. The ultimate aim of this mechanism is a faithful duplication and even segregation of the genetic material and of the organelles between the cells. To achieve this, all the steps of the cell cycle must follow one another in an exquisitely precise way that grants for example that chromosomes are replicated only once per cell cycle and that the replication of the DNA is completed before the onset of nuclear segregation.

Saccharomyces cerevisiae cell cycle

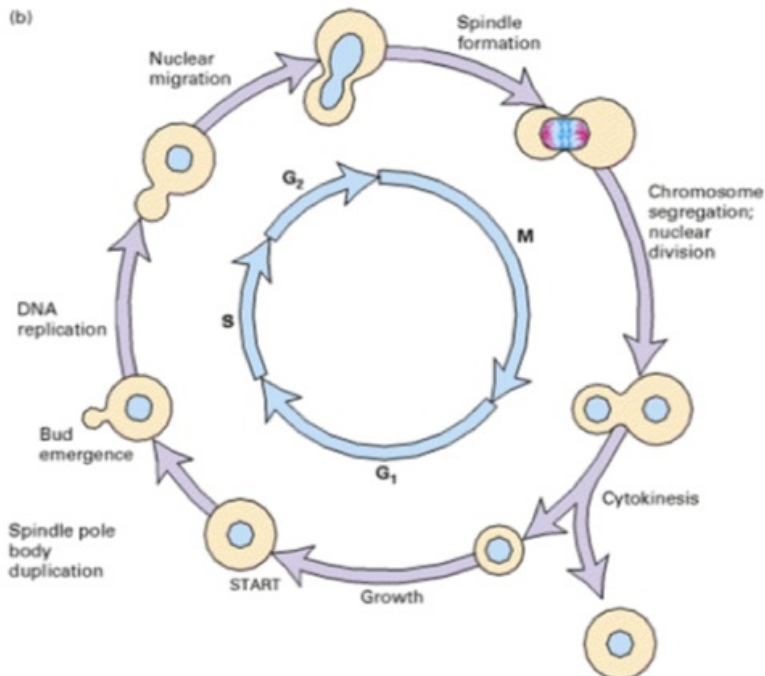


Figure 11. Haploid cell cycle of the budding yeast *Saccharomyces cerevisiae*¹

The unicellular fungus *Saccharomyces cerevisiae* has proven to be an invaluable model organism thanks to a fast duplication time, a well-known genetics and to conservation of biological features with higher eukaryotes. This organism can be found in two states: an haploid one, which is characterized by two sexes, MAT_a and MAT_α, and a diploid one, MAT_a/α. Budding yeast cells can shift from one of these states to the other: when two cells of opposite mating type meet, they can mate and generate a diploid strain that in turn, upon lack of nutrients, can undergo meiosis and form four haploid spores held in a ascus, which protects them from heat and desiccation. A typical haploid cell cycle (fig.11) begins in G1 with a single unbudded cell which contains a complete set of chromosomes and a single Spindle Pole Body (SPB, the microtubule organizing center of budding yeast). If the environment satisfies the requirements for cell duplication, cells are prevented from entering the G0 and instead commit the cell cycle. This process begins when cells proceed through the START, a point after which the cell cycle can not be interrupted, and trigger three fundamental events for the S phase: SPB duplication, replication of the genetic material and bud emergence. The main focus of the S phase is the complete and faithful duplication of the genome, which occurs along with bud growth. During G2 the SPBs split and the nucleus is pulled towards the interface between the mother and daughter cells, namely the bud neck. Finally, in M phase, the cohesin complexes that hold sister chromatids together are cleaved, chromosome segregation is triggered and two separate cells are generated through cytokinesis.

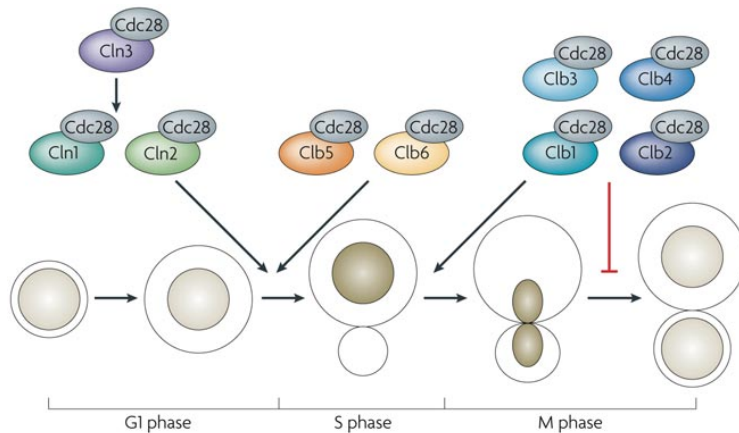


Figure I2. Cyclins in mitotic budding yeast cell cycle²

CELL CYCLE REGULATION

The success of the cell cycle and hence of cellular reproduction is strictly dependent on the fact that all its key features occur in a strictly precise order and timing. This is ensured by a family of proteins named Cyclin Dependent Kinases (CDKs) that, interacting with different classes of regulatory proteins called cyclins, earn specificity for different substrates promoting distinct events of the cell cycle. Budding yeast genome codes for six CDKs: Cdc28, Pho85, Kin28, Srb10, Bur1 and Ctk1. The only essential CDK of this organism, also known as CDK1, is Cdc28, while other CDKs regulate secondary processes³⁻⁵. To ensure a rapid response to external stimuli and grant that cell cycle progression occurs in a fast and robust way, the concentration of CDKs is not heavily modulated along the cell cycle, and exceeds that of cyclins⁶. This means that the interaction between a CDK and a cyclin and the series of events caused by this interaction can only happen depending on the availability of that cyclin in a particular moment of the cell cycle⁶. Levels of cyclins strongly oscillate during the cell cycle, particularly thanks to extremely regulated expression and degradation patterns⁶. Different classes of cyclins are present in budding yeast (fig.I2): G1 cyclins (Cln1, Cln2 and Cln3, necessary for the

beginning of the cell cycle), and type B cyclins, divided in S phase cyclins (Clb5 and Clb6, involved in DNA replication) and M phase cyclins (Clb1-4, required for the assembly and function of the mitotic apparatus)⁷.

REGULATION OF CELLULAR PROLIFERATION

Passing through START, cells enter a cell cycle that cannot be interrupted, and hence commitment of the cell cycle is a tightly regulated decision. Two mechanisms prevent unscheduled cell cycle commitment: on one hand two Cyclin dependent Kinase Inhibitors (CKIs, namely Sic1 and Far1) physically interact with Cdc28 and keep it in an inactive state prior to the START, and on the other hand inhibition of a protein complex made up of Swi4/6 and known as the SBF by Whi5 prevents *CLN1* and *CLN2* transcription⁷⁻⁹.

The sequence of events leading to START in budding yeast was first assessed by Hubler and colleagues in 1993¹⁰. They exploited cells of a particular background that allowed an easy modulation of cellular cyclic-AMP (cAMP) levels, starving cells and adding then either nutrients or cAMP to induce cell proliferation. With this experimental setup the authors observed that there was first an increase in the levels of Cln3, dependent on protein synthesis and for which the cAMP pathway was dispensable. This first stage was followed by another one that was instead sensitive to cAMP levels and promoted further Cln3 accumulation and sustained *CLN1* and *CLN2* expression¹⁰. The enzyme responsible for cAMP production is adenylate cyclase (AC), Cyr1 in budding yeast, whose activity is tightly regulated by the small GTPase Ras¹¹. Ras-GTPases are ubiquitous essential proteins in eukaryotic cells, where they play a fundamental role in cell cycle regulation and, noteworthy, Ras signalling is altered with a significant incidence in several types of human cancers¹². The main role of Ras proteins, Ras1 and Ras2 in budding yeast, is to couple cell cycle commitment to nutrients availability by promoting Cyr1 activity^{13,14}. In

this scenario, Ras is part of an intricate network ascribed to translate signalling from two glucose-sensing modules in AC activation and hence cell cycle progression. Activity of Ras in this pathway seems to be mediated by some intermediates in glucose processing and is modulated by two GAPs (Ira1 and Ira2) and two GEFs, the essential Cdc25, and the dispensable Sdc25, which only takes part in Ras activation upon growth on poor media (fig.I3)¹⁵⁻²¹. Ras regulation likely occurs before plasma membrane (PM) delivery, as Cdc25, Ira1 and Ira2 are mainly localized to ER and mitochondria, respectively, and another level of modulation of this pathway is hence provided by spatial regulation of its players²². However, it has been demonstrated that Cyr1 could act as a scaffold protein for Ras and its GAPs on the cell membrane to dampen Ras signalling in a AC activity independent manner^{23,24}. Accumulation of Ras on the PM occurs thanks to irreversible farnesylation of its terminal CAAX motif, followed by reversible palmitoylation by the Erf2/Erf4 complex^{25,26}. The actual mechanism leading to PM binding has not been completely understood: a dual model in which both secretory pathways as well as a direct role for Erf2/Erf4 has been proposed, but unveiling the exact contribution of the two branches is made challenging by the requirement of Erf2/Erf4 activity for stable membrane interaction^{25,27-29}. Moreover, depalmitoylation and recycling of Ras through the cytoplasm has been observed and a role for vacuolar proteins and mitochondria has also been reported^{30,31}.

GTP-bound Ras triggers Cyr1 to synthesize cAMP and in turn this metabolite relieves a protein complex, the Protein Kinase A (PKA), from its inhibitory subunit Bcy1, promoting cell cycle progression³². Due to the delicate feature it promotes, this mechanism of cAMP production has to be tightly regulated, to avoid deleterious cell cycle commitment in lack of nutrients and indeed loss of genes that prevents cell cycle exit in poor media is reported to cause a Ras-dependent cell death³³. For this reason cells have evolved a feedback

mechanism to dampen cAMP levels based on active-PKA itself that triggers the low- and high-affinity phosphodiesterases Pde1 and Pde2 at the level of posttranslational modifications, protein abundance and localization to degrade cAMP³⁴⁻³⁷. As mentioned, the ultimate goal of the cAMP is activation of the PKA, a heterotetrameric complex made up of Tpk1-3 and two subunits of Bcy1^{38,39}. Active PKA deeply manipulates cellular behaviour, promoting features associated to rapid growth and inhibiting others linked to stationary phase. The protein complex acts at the level of posttranslational modifications of its targets and altering gene expression. Passing through the START is inhibited by Whi3, a RNA-binding protein that acts as a negative regulator of cell cycle progression by sequestering Cln3 mRNA in cytoplasmic foci and preventing nuclear accumulation of Cdc28-Cln complexes^{40,41}. Active-PKA phosphorylates Whi3 on Ser-568 inhibiting it and thus promotes an increase in Cln3 levels which triggers degradation of the CKIs⁴².

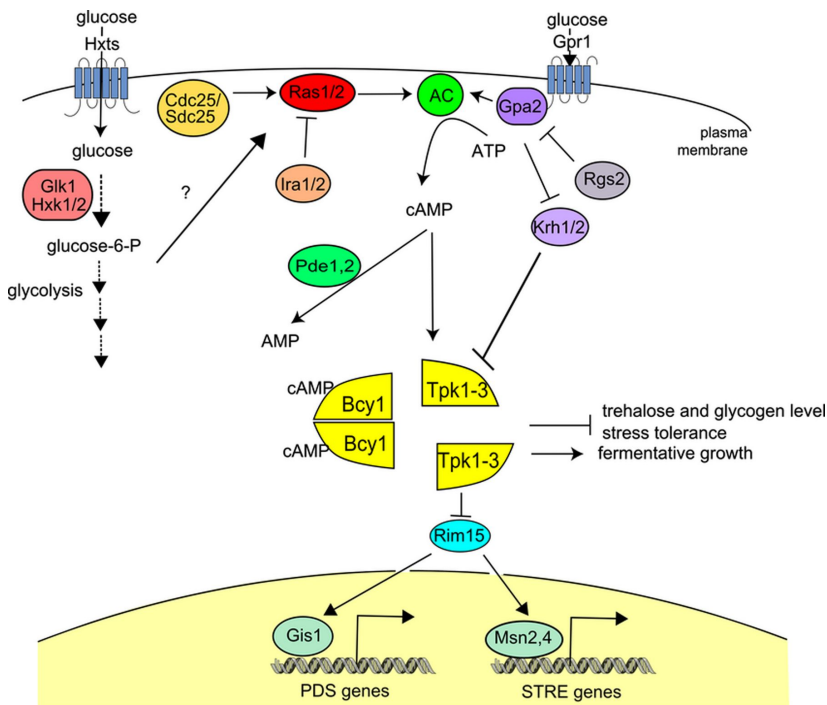


Figure I3. PKA activation pathways⁴³

Moreover, Cln3-Cdc28 complexes phosphorylate Whi5 and trigger the activity of the SBF. Though the regulation of this mechanism is not completely clear, it has recently been demonstrated to couple cell cycle progression with the presence of a functional vacuole, as cells lacking or deficient in this organelle accumulate Whi5⁴⁴. Vacuoles are budding yeast equivalent of lysosomes, double-membrane enclosed organelles with a plethora of functions ranging from degradation of macromolecules to autophagy, aminoacid storage and traffic⁴⁵. In budding yeast their size and shape are highly regulated according to the growth medium and the cell cycle stage. The main regulator of vacuole size and shape is a protein complex located on the vacuolar membrane composed of Fab1, Fig4, Vac7 and Vac14⁴⁶⁻⁴⁸. Fab1 is a phosphatidylinositol (PtdIns) 3-phosphate 5 kinase that converts PtdIns(3)*p* to PtdIns(3,5)*p*₂ in a reaction reversed by the phosphatase Fig4. Vac7 and Vac14 have structural roles and are required, along with Fig4, for Fab1 activity⁴⁷⁻⁴⁹. Loss of Fab1 activity has been related to vacuole enlargement, defective post-engulfment nutrient recovery, decreased migration and invasion and nuclear missegregation (Fab1 stands for Forms Aploid and Binucleated)^{46,50-52}. As mentioned, vacuole size and shape are coupled to different cell cycle stages and a single, round vacuole of the right size must be present to allow Whi5 inhibition and hence promote Cln3-CDK-mediated *CLN1* and *CLN2* expression^{44,49}.

The newly assembled Cln1-Cdc28 and Cln2-Cdc28 complexes further phosphorylate Whi5, resulting in a strong positive feedback that causes a burst in Cln levels^{53,54}. Accumulation of Clns promotes three pivotal events in budding yeast cell cycle, SPB duplication, DNA replication and bud emergence.

Increase in Cln-CDK levels is required for SPB replication, however an unrestricted Cln accumulation, as obtained by overexpression of Cln2 in a *clb1-5Δ* strain, results in an unscheduled and detrimental re-replication of the SPBs

that are however not separated⁵⁵. Progression of the cell cycle and the inhibitory effect of Clb5-6 on Cln levels ensure that the SPB is duplicated only once per cell cycle and promote SPBs separation⁵⁵. Accumulation of Clb5 and Clb6 is also required to start the replication of the genetic information^{7,9}. Due to the extremely delicate features they promote, Clb5 and Clb6 are tightly regulated and prevented from interacting with CDKs during other phases of the cell cycle thanks to CKIs (Cyclin-Kinase Inhibitors)^{7,8}. These inhibitors are primed for ubiquitylation and subsequent degradation by a Cln-Cdc28-mediated phosphorylation⁵⁶⁻⁵⁸. Promotion of S phase is synonym with inhibition of G1 cyclins: Clb-Cdc28 activity promotes transcription of *CLB1* and *CLB2* and triggers a Whi5-mediated repression on Cln genes⁵⁹.

POLARIZED GROWTH IN BUDDING YEAST

Budding yeast enters the cell cycle as a single, round cell that develops in a so-called polarized growth to emit a bud in late G1 that will increase in size and then pinch-off the mother through cytokinesis (fig.I4). This polarized pattern in which material deposition and cell growth are directed towards specific compartments of the cell periphery is however common to all cells and research in this field is of pivotal importance as polarity de-regulation may lead to severe diseases and is one of the first steps of carcinogenesis⁶⁰.



Figure I4. Morphological changes in budding yeast cell cycle⁶¹

Polarized growth is controlled in all eukaryotes by the small, essential GTPase Cdc42 and its regulators^{62,63}. In budding yeast, Cdc42 oversees cellular growth and shape by modulating polarization, actin cytoskeleton, assembly of the septin-ring, vesicle dynamics and mating by interacting and triggering a plethora of different effectors^{62,64–66}. To orchestrate all its functions, the activity of Cdc42 has to be tightly modulated during the cell cycle, and this task is achieved thanks to four GAPs (Rga1, Rga2, Bem2 and Bem3), a GDI (Rdi1) and a single, essential GEF (Cdc24)(fig.I5)^{67–72}. Noteworthy, both GAPs and the GDI play a positive role in establishing clusters of active-GTPase^{73,74}.

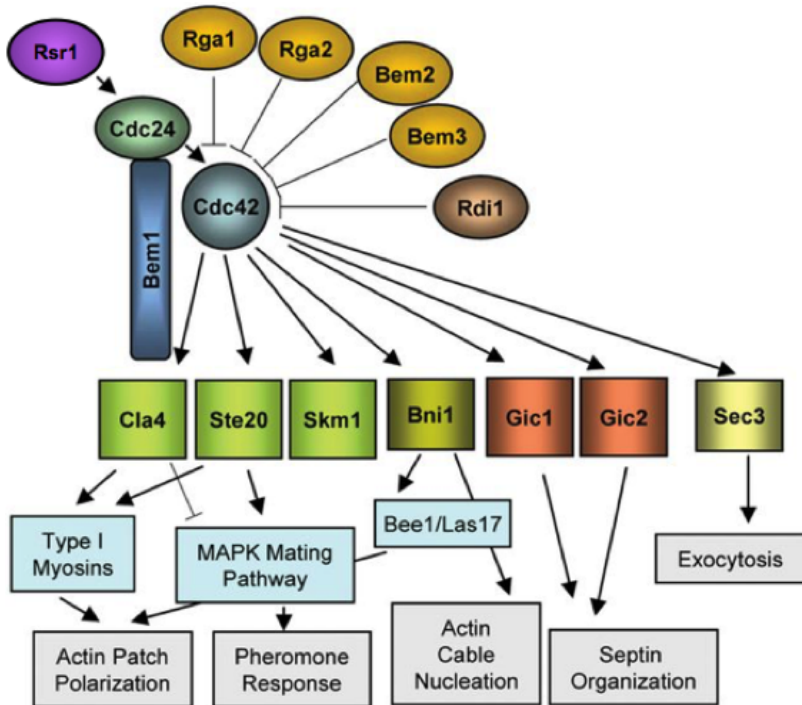


Figure I5. Cdc42 regulation and functions (modified from)⁷⁵

Cdc42 is first required in early G1 to drive bud emergence. An absolute requisite for this event is the formation of a localized accumulation of the GTPase that is promoted either through a actin-dependent delivery of Cdc42 at

the presumptive bud site (though this hypothesis is currently challenged by another model based on Cdc42 recycling) or in an actin-independent mechanism which requires the scaffold protein Bem1 to assemble a Cdc42-signaling complex^{73,74,76,77}. A key event in this mechanism is the accumulation of Cdc24 at the presumptive bud site and to sites of polarized growth in G1 and S/M phases respectively to be then sequestered into the nucleus by its interactor Far1 in late mitosis and until entry in the next cell cycle^{73,78,79}. To successfully localize at polarity sites in G1, Cdc24 requires Cln1,2-Cdc28 activity, likely because it is necessary for Far1 degradation and hence for a strong release of Cdc24 from the nucleus⁸⁰. Clns also play a role in Rga2 inhibitory phosphorylation by CDK and mutation of Rga2 phosphosites as well as defective CDK mutants cause lowered Cdc42-GTP levels⁸¹. Proper distribution of the GEF in G1 is ensured by its physical interaction with the Ras-family GTPase Rsr1 and the scaffold protein Bem1. Rsr1 recruits Cdc24 in early G1 concomitantly with Whi5 being still present in the cell⁸². This pathway of Cdc24 clustered activation requires Bud5, the Rsr1 GEF, to produce GTP-Rsr1, which is in turn able to physically interact with Cdc24 and recruit it⁸³. Then, the Rsr1 GAP Bud2 hydrolyzes GTP-Rsr1 and triggers the activation of Cdc24⁸⁴. On the other hand, Bem1 is a scaffold protein that physically interacts with a Cdc42-GTP effector (PAK) and Cdc24 and so promotes a positive feedback loop in which clustered active-Cdc42 recruits Bem1/Cdc24 complex and favors GTP loading of surrounding Cdc42-GDP (fig.I6)⁸⁵⁻⁸⁷.

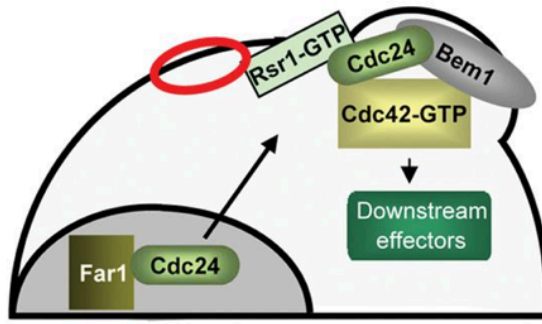


Figure I6. Mechanisms of Cdc24 spatial control⁷⁵

An additional contribution in building a cluster of active-Cdc42 is provided by the GDI Rdi1 that traps Cdc42-GDP and transfers it from the PM into the cytoplasm⁸⁸⁻⁹⁰. Deletion of both *RSR1* and *BEM1* is reported to be lethal, however some viable *rsr1Δbem1Δ* strains have been obtained, suggesting the existence of yet other mechanisms that may partially take part in Cdc24 polarization^{77,86,91}. Evidence in other organisms indicate that Ras may be involved in Cdc42 regulation and that it physically interacts with Cdc24, though the relevance of this interaction has not been unveiled^{92,93}. These observations however rise the possibility that what allows some *rsr1Δbem1Δ* cells to polarize Cdc24 and proliferate could be Ras.

A big challenge in cellular duplication is a peculiar distribution and maintenance of cell determinants that allow a different behaviour in mothers and daughters. This is possible thanks to physical diffusion barriers assembled at the mother/daughter interface that prevent material exchange between the two compartments. In budding yeast this barrier is provided by a ring-like structure composed of the so-called septins, a class of GTPases that is able to assemble heterocomplexes in rod- or ring-like structures^{94,95}. Four essential (Cdc3, Cdc10, Cdc11 and Cdc12) and one dispensable (Shs1) septins are present in budding yeast, where they are assembled in a ring structure at the presumptive bud-site⁹⁶. The process of ring formation and maturation into a

collar and then double ring has not been unveiled in detail, but it is known to have strong interplays with Cdc42. A tripartite mechanism where Cdc42-GTP directs vesicle delivery, promotes septin assembly and is in turn inhibited by septins accounts for bud emergence has been described. First, a stable cluster of Cdc42-GTP is built and directs septin recruitment thanks to a direct physical interaction between its effectors Gic1 and Gic2 and septins⁹⁷. Being Cdc42-GTP accumulated in a cap, septins would likely assemble in a similar structure. This is prevented by two other mechanisms. On one hand, septins trigger Cdc42-GAP Rga2, resulting in a local inhibition of the GTPase⁹⁸. On the other hand, actin cytoskeleton (whose orientation relies on Cdc42 activity as described below) points towards the centre of the Cdc42-GTP cluster and deposition of new material is directed to the same compartment. Overall this promotes the generation of a hollow septin cap, restricting septin deposition only at cluster borders and generating a ring. Once the ring is formed, septin restricts Cdc42-GTP in the bud and ensure its proper growth (fig.I7)⁹⁸.

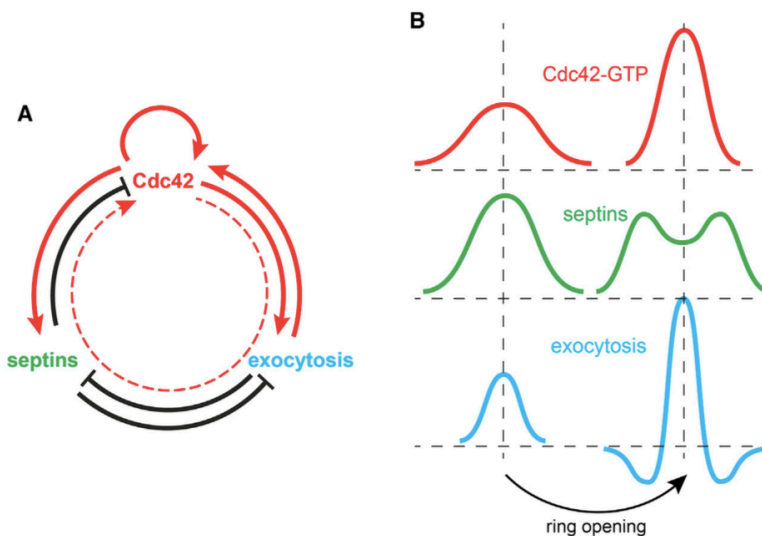


Figure I7. Schematic representation of Cdc42, exocytosis and septins interplay (A) and septin cap to ring transition (B)⁹⁸

Once SPBs are duplicated, DNA is replicated and the bud is emitted, cells enter a phase during which the daughter apically grows to approach the dimension of a mature cell. Cdc42 promotes the morphological changes required for cellular growth through a constant reorganization of the actin cytoskeleton, a network made up of actin cables that is widely exploited by cells to carry out several key functions, such as the delivery of different organelles and is therefore essential for viability (fig.I8)⁹⁹⁻¹⁰¹.

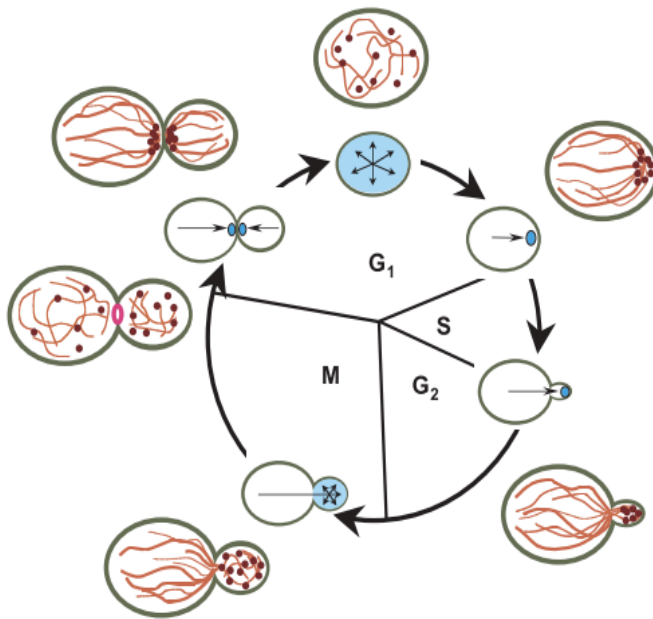


Figure I8. Actin cables organization and cell growth through the cell cycle¹⁰²

Actin cables are F-actin polymers organized in linear bundles nucleated and elongated by so-called formins. Budding yeast genome codes for two formins, Bnr1 and Bni1, that act downstream of Rho GTPases and are responsible for the synthesis of different cable populations in the cell¹⁰³⁻¹⁰⁵. Bni1 is responsible for the random orientation of cables in unbudded cells, while after budding it is accumulated at the bud tip, from where it organizes the cytoskeleton of the daughter cell; Bnr1 is on the other hand present only at the bud neck and

elongates cables in the mother cell¹⁰⁶. Overall, this organization of the cytoskeleton accounts for material transport towards the daughter cell¹⁰⁷. Late in mitosis Bnr1 is displaced from the bud neck and replaced by Bni1, thanks to the Cdc42 effector Spa2 which mediates Bni1 recruitment¹⁰⁸⁻¹¹⁰. Bnr1 and Bni1 differ in their motility as determined by their exchange ratio from the bud neck and bud tip respectively, measured by FRAP studies that demonstrated how Bnr1 is stably attached to the septin ring, while Bni1 dynamically exchange between the bud cortex, actin cables and the cytoplasm^{106,111,112}. Both Bnr1 and Bni1 structures comprise a RBD (Rho Binding Domain) supposed to autoinhibit formin activity, a FH1 domain that, interacting with profilin, facilitates actin delivery to formins and a FH2 domain responsible for actin nucleation activity^{103,105,113-115}. Formin autoinhibition can be relieved by binding of Rho3 or Rho4 to the RBD, and loss of both these GTPases causes cell death as the outcome of a failure in building a functional cytoskeleton^{116,117}. In this scenario, Cdc42 is dispensable for actin cable assembly, but pioneering experiments performed on conditional mutants showed how its activity is essential to regulate the proper spatial organization during budding and growth, allegedly regulating formin distribution¹¹⁷. To promote efficient cable synthesis, the activity of formins can be enhanced by the actin nucleation promoting factor Bud6, which interacts with and enhances the activity of Bnr1 and Bni1 and deletion of *BUD6* results in an altered budding pattern¹¹⁸⁻¹²¹. The localization of Bud6 varies during the cell cycle: it is first enriched at the presumptive bud site and then at the bud tip until metaphase, when it starts to accumulate also at the bud neck. During cytokinesis it is only detectable at the mother-daughter interface^{120,122}. This peculiar localization pattern relies on formins, with Bud6 interacting with both Bnr1 and Bni1 and being hence recruited at the bud neck and bud tip (fig.I9)¹²³.

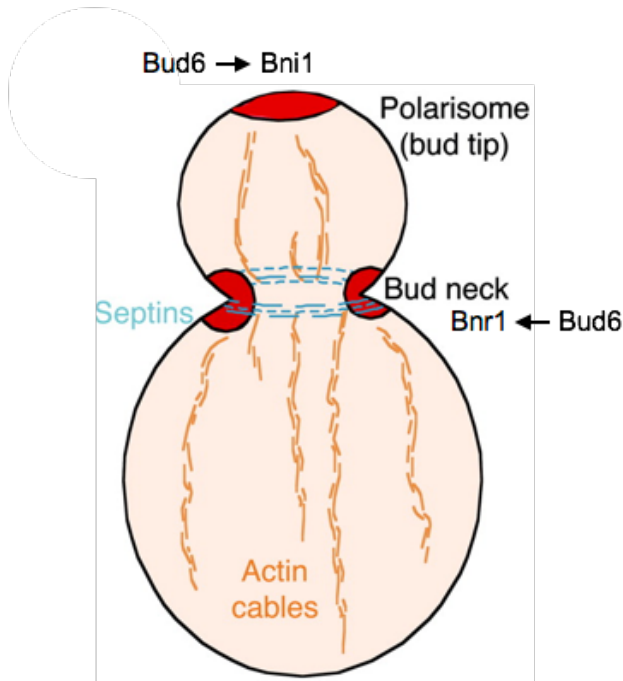


Figure I9. Bni1, Bnr1 and Bud6 spatial organization (modified from)¹²⁴

VESICLE TRAFFICKING

One of the main functions of the actin network is the delivery of proteins, lipids and other molecules to the PM, which together promote cell growth. This delivery is performed by vesicles that can generate from any membrane-enclosed organelle and travel along actin cables to unload their cargo to different cellular compartments.

Two main protein complexes take part in vesicle delivery and fusion with target membranes, the exocyst and the SNARE complexes. The exocyst is made up of Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84, and its function is to determine the sites of vesicle docking and fusion¹²⁵. The differential distribution of the bricks of this complex allows the preferential delivery of vesicles to particular membrane departments. Six members (Sec5,6,8,10,15 and Exo84) accumulate on vesicle membrane, while Sec3 and Exo70 are located on target membranes and mark them as acceptor domains (fig.I10)¹²⁶. The

distribution of Sec3 and Exo70 hence accounts for preferential delivery of vesicle to particular clusters on target membranes. In this scenario, besides directing actin cable organization, Cdc42 also plays a direct role in traffic regulation by directly interacting with the N-term of the exocyst member Sec3 and disruption of Cdc42-Sec3 interaction blocks exocytosis^{127,128}. Sec3/Exo70 recruitment on membranes also relies on the presence of Pleckstrin Homology (PH) domains in their sequence that enable them to interact with PtdIns(4,5)*p*₂, which thus acts as a marker for vesicle delivery sites¹²⁵. Once a vesicle approaches a Sec3-Exo70 decorated membrane, a complete exocyst complex is assembled and primes the vesicle for the fusion event which requires the formation of a functional SNARE complex¹²⁹.

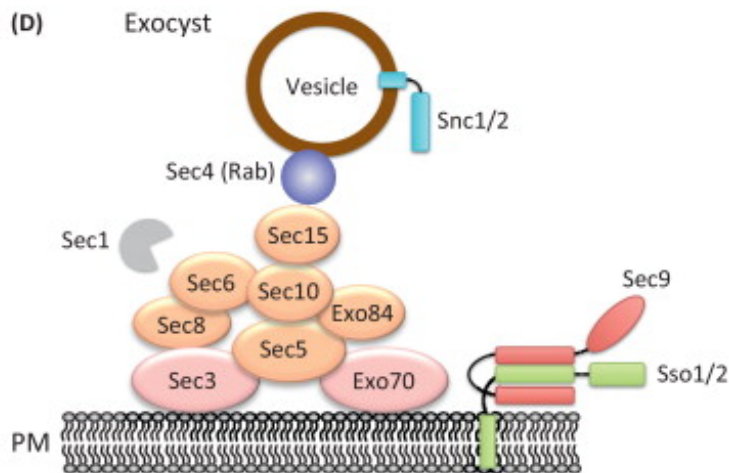


Figure I10. Exocyst complex¹³⁰

Thanks to the morphological changes promoted by Cdc42 and its effectors, the bud enlarges and organizes its actin cytoskeleton to fulfil all the requirements for a correct nuclear segregation and cell division that will take place in mitosis.

SACCHAROMYCES CEREVISIAE MITOSIS

During mitosis cells have to undergo a faithful and even segregation of their genetic material and organelles into daughter cells before the onset of cytokinesis, making this cell cycle phase critical. Entry in M phase is promoted by specific B-type cyclins (Clb1-4). These proteins are prevented from reaching deleterious levels during interphase thanks to a very low transcription rate and efficient degradation and drive the cell through mitosis and cytokinesis before being rapidly degraded to allow entry in a new G1 phase¹³¹⁻¹³⁵.

Several substages (prophase, prometaphase, metaphase, anaphase and telophase) follow one another during mitosis to achieve the different requirements needed for a proper cellular division. The first step in mitosis is chromatin condensation in pairs of sister chromatids with a rod-like structure, which are held together by the cohesin complex, whose subunits are Smc1, Smc3, Scc1 and Scc3. Once DNA condensation is completed, the nuclear envelope breaks down during prometaphase to allow the establishment of microtubule-chromatid interactions. In *S.cerevisiae*, however, nuclear envelope breakdown is not required thanks to its peculiar SPBs, which are embedded in the nuclear membrane¹³⁶. Each sister chromatid has a large protein complex, named kinetochore, attached to its centromeric region, whose essential role is the binding of microtubules emanating from the MTOCs¹³⁷. When the sisters of a pair are attached by microtubules emanating from two different MTOCs, a configuration suitable for successive segregation is achieved¹³⁸. The interaction between microtubules and kinetochores is mediated by the KMN network, a complex with structural functions made up by Spc105 and two other subcomplexes named Mis12 and Ndc80, which are all essential for a functional microtubule attachment^{139,140}. The microtubule/kinetochore interaction is initially highly dynamic, and it often occurs between the side of a microtubule and the kinetochore so that the microtubule has to slide along the chromosome

until it finally interacts with the kinetochore with its plus end¹⁴¹. At this point, the resulting pulling force on the attached kinetochore promotes the orientation of the unattached one towards the opposite MTOC, facilitating the achievement of a correct amphitelic configuration¹⁴¹.

The functional amphitelic attachment is not always immediately reached, as the search and capture mechanism for attachment is stochastic. A successful segregation of the genetic material hence requires a proper coupling of the nuclear dynamics with cohesin degradation, so that correct microtubule/kinetochore interactions are established before cleavage of Scc1 to prevent unscheduled cohesin degradation in presence of misattachments that would result in chromosome missegregation and aneuploidy. Cells have thus evolved a surveillance mechanism to avoid deleterious anaphase onsets until all chromosomes-kinetochores interaction are amphitelic. The SAC (Spindle Assembly Checkpoint), perceives the presence of misattached kinetochores and prevents cohesin degradation until a functional mitotic spindle is assembled¹⁴².

The SAC network in *S.cerevisiae* is made up of six proteins that accumulate on the outer side of unattached kinetochores: Mad1-3, Mps1, Bub1 and Bub3^{143,144}. The actual signal that primes SAC activation has still to be unveiled, but two hypotheses have been raised and, allegedly, coexist. A first model proposes that the SAC may perceive unattached microtubules detecting the occupancy of kinetochores, while another model claims rather that the SAC is able to detect lack of tension on kinetochores when they are not bound to microtubules¹⁴⁵⁻¹⁴⁷. Whatever the signal that primes for SAC activation, a prominent role in triggering the checkpoint effectors is performed by Ipl1, the Aurora B kinase of budding yeast¹⁴⁸. This protein is part of a larger complex known as the Chromosomal Passenger Complex (CPC), along with INCENP, Survivin (which recognizes H3-Thr3 phosphorylation) and Borealin (which is recruited by P-H2AS121-bound Shugoshin) and has the role to oversee a

correct microtubule attachment, triggering the SAC and thus preventing anaphase onset in case of misattachments^{149–151}. Upon its recruitment to centromeric regions, the CPC promotes phosphorylation of CENP-A and key subunits of the kinetochore promoting two key events for faithful chromosome segregation. First, in a still unveiled way Aurora B mediates the recruitment of SAC protein Mps1, which in turn stimulates recruitment of other subunits of the checkpoint¹⁵². Second, Aurora B mediated phosphorylation of kinetochore components renders the microtubule-kinetochore interaction less stable thus facilitating detachment and new microtubule capture providing a chance to achieve a correct configuration¹⁴⁸. Once the SAC has been triggered, it inhibits anaphase onset sequestering and stimulating the degradation of Cdc20, a fundamental protein for Scc1 degradation (fig.I11)^{153–156}.

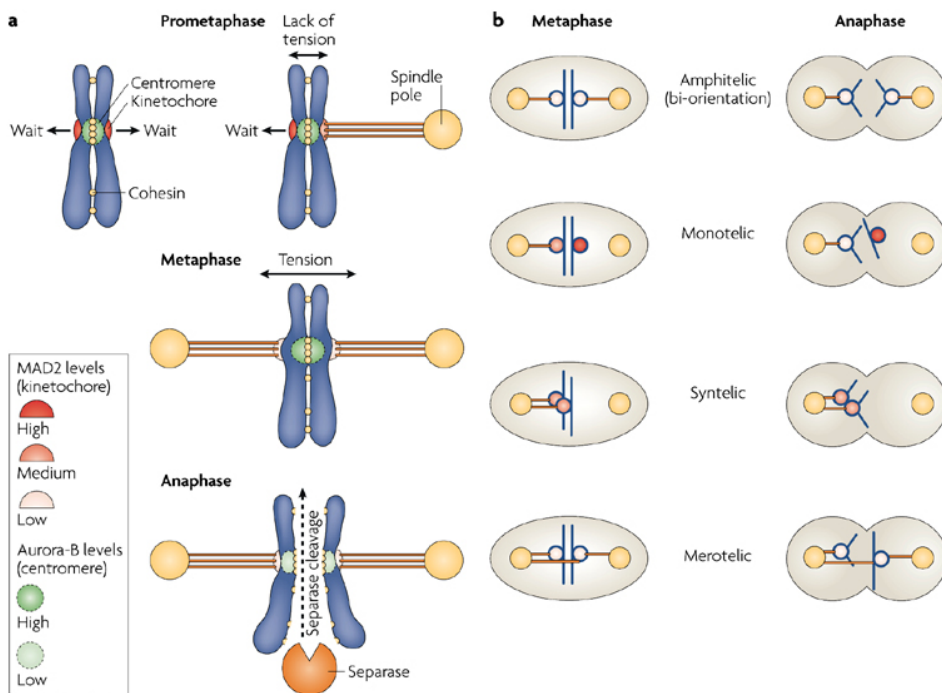


Figure I11. SAC activation by misattached kinetochores¹⁴²

When all chromosomes have been successfully aligned, the SAC is turned off and metaphase to anaphase transition is triggered¹⁵⁷. At this stage, progression of the cell cycle is regulated by the activation of a large protein complex with E3 ubiquitin ligase activity, the Anaphase Promoting Complex (APC/C). APC/C is not able to act as an E3 per se, rather it requires interaction with one of two other proteins, Cdc20 and Cdh1, that act as specificity factors to allow it to physically interact with its targets¹⁵⁸⁻¹⁶¹. The essential feature of Cdc20 is to promote the APC/C-dependent cleavage of the cohesin complex (although it is also involved in degradation of other proteins such as M-phase cyclins) and due to the extreme delicate function it promotes, its levels oscillate during the cell cycle with a prominent accumulation in mitosis and subsequent rapid degradation in G1^{158,161-167}. Besides Cdc20 levels, formation of the APC/C^{Cdc20} complex is further regulated by a Clb-Cdc28-mediated phosphorylation of the APC/C core subunits that renders the complex able to bind Cdc20¹⁶⁸. Once the APC/C^{Cdc20} complex is assembled, it primes for degradation the protein Pds1, known as securin, which acts as a physical inhibitory of Esp1, the protein responsible for Scc1 cleavage promoting anaphase onset¹⁶⁹⁻¹⁷¹. When sisters have been separated, another APC/C complex (APC/C^{Cdh1}) primes Cdc20 for degradation (fig.I12)¹⁶⁷.

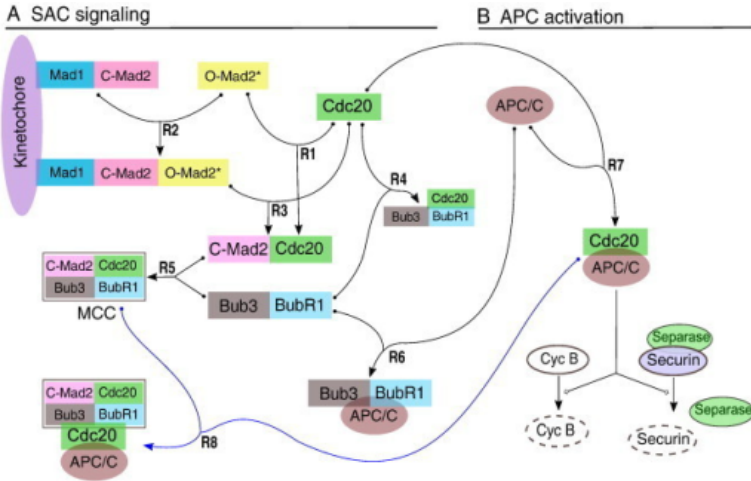


Figure I12. Mechanisms of SAC-mediated anaphase prevention and coupling to cohesin degradation¹⁷²

Once cohesins have been degraded, nuclei can be pulled apart and segregation follows the orientation of mitotic apparatus.

Orientation of the spindle depends on microtubule organizing centers (MTOCs) of the cell, whose positioning is regulated through interactions by astral microtubules^{173–177}. Two pathways named the early and the late pathway regulate nuclear localization during mitosis, with the overall aim to position the nuclei in an optimal site for their subsequent segregation (fig.I13).

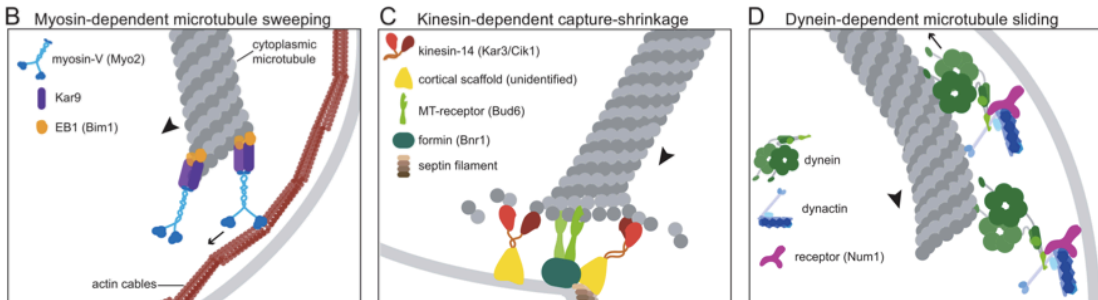


Figure I13. Different mechanisms of nuclear positioning regulation in budding yeast (modified from: ¹⁷⁸)

The early pathway is active prior to anaphase and in early stages of this phase and most of the actors involved in this branch are localized to the bud cortex¹⁷⁸. In this mechanism, the alignment of the mitotic spindle along the mother bud axis is promoted through two different branches. In the first branch, the myosin Myo2 exploits actin cables as tracks along which it sweeps microtubules plus end towards the bud tip. Myo2/microtubule tip interaction is bridged by Kar9 and the motor protein Bim1, which travels along microtubules towards their plus end carrying Kar9 and, once at the tip of the microtubule, Kar9 binds Myo2^{179–182}. Myo2 then, traveling along actin cables, pulls the microtubule, along with the SPB and the nucleus, towards the daughter cell¹⁷⁸. The other branch of the early pathway exploits coupled microtubule capture on the cell cortex and shrinkage. In this case, Bud6 acts as a cortical receptor for microtubules, and the mechanism underlying microtubule capture/shrinkage are still unknown, though it has been shown that this pathway is active only towards the bud neck and the bud tip^{176,183}.

The late nuclear positioning pathway relies on the motor protein dynein to promote proper spindle orientation and cells deficient for dynein activity experience a delay in cell cycle progression due to a temporary failure to pull a nucleus in the daughter cell^{184–186}. Again, in this mechanism the plus end of a microtubule primes the cell cortex searching cortical capture sites which are here represented by Dynein interactor Num1. Dynein is transported to microtubule plus ends by Bik1 and upon interacting with Num1 on the cell cortex, it is transferred to the cortex along with the dynactin complex. At this point, the motor activity of dynein is triggered, promoting microtubule sliding and generating pulling forces on the nucleus that segregate chromosomes^{187–191}. APC/C^{Cdc20} mediates the degradation of a fraction of securin, which is still present through anaphase and inhibits further cell cycle progression¹⁹². The complete degradation of Pds1 requires the formation of another protein

complex, APC/C^{Cdh1}^{193,194}. Cdh1, is regulated mainly at its protein levels, which rise from late mitosis to G1, when it exerts two main roles promoting degradation of Cdc20 and B-type cyclins^{167,195,196}. Degradation of Cdh1 during interphase is dependent on APC/C^{Cdh1} itself and CSF, another ubiquitin ligase complex^{197,198}. The activity of the APC/C^{Cdh1} complex is also regulated by posttranslational modifications, as Cdh1 is target of an inhibitory phosphorylation that prevents it to interact with APC/C and occurs during S and M phases and is removed in late mitosis and G1^{163,199,200}. The shift from APC/C^{Cdc20} to APC/C^{Cdh1} is regulated by the essential phosphatase Cdc14, which on one hand directly dephosphorylates Cdh1 rendering it able to bind the APC/C and on the other hand inactivates Clb/CDK complexes responsible for Cdc20 loading on the APC/C^{168,201-203}. Newly assembled APC/C^{Cdh1} completes securin degradation and targets several other proteins among which is Clb2, thus inhibiting Clb/CDK complexes (fig.I14)¹⁹².

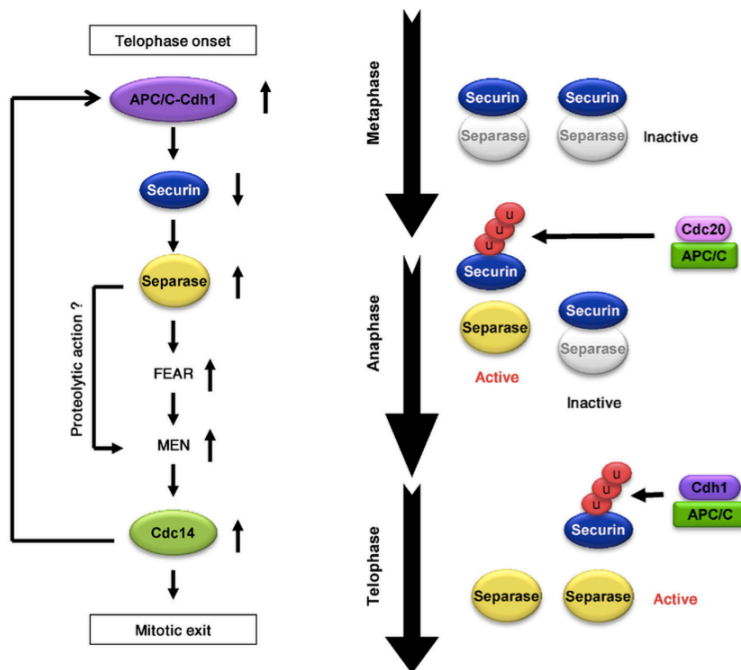


Figure I14. Securin function at the anaphase/tephase transition¹⁹²

Prior to mitotic exit, Cdc14 activity is prevented by its sequestration in the nucleolus thanks to its physical interactor Cfi1/Net1^{204,205}. Relieving of the phosphatase from its inhibitor and its following release in the cytoplasm are promoted by the Cdc14 Early Anaphase Release (FEAR) and the Mitotic Exit Network (MEN) (fig.I15). The former has the function to release Cdc14 in the nucleus in early anaphase and is dispensable for cell cycle progression, while the MEN promotes the release of Cdc14 in the cytoplasm and is essential. Apart from APC/C^{Cdh1} complex formation, Cdc14 facilitates mitotic exit acting on several other targets. Cdc14-mediated Sic1 accumulation promotes Clb/CDK inactivation²⁰⁶. Other targets of the phosphatase are Bnr1 and Bni1, eventually affecting formin localization¹¹⁰. Finally, Cdc14 directly promotes cytokinesis dephosphorylating Inn1 and the essential protein Iqg1, which acts as a scaffold protein to promote actomyosin ring contraction at the bud neck^{207–209}

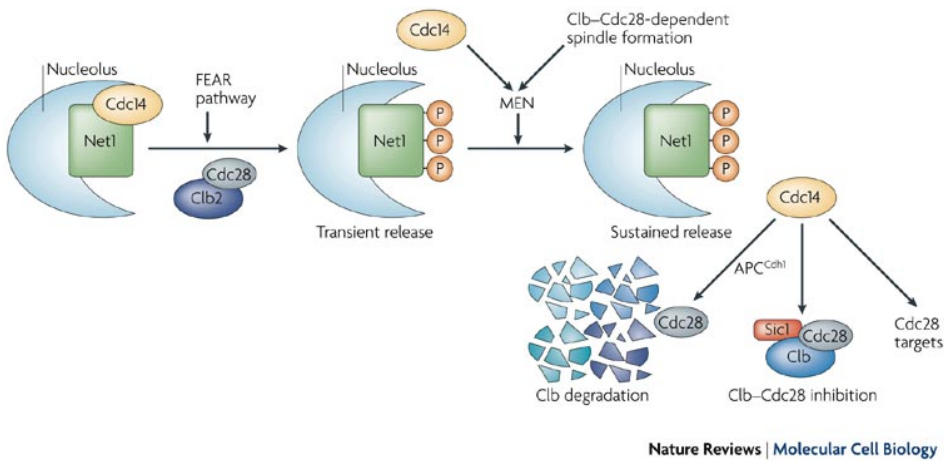


Figure I15. Cdc14 activation and function²

If Cdc14 activity is triggered in presence of a non-properly aligned spindle, cells may experience unbalanced chromosome segregation and aneuploidy following cytokinesis. This is prevented by the SPOC, a surveillance

mechanism that inhibits Cdc14 and hence mitotic exit in case of spindle misorientations (fig.I16)^{183,210}. Proper orientation of the mitotic apparatus is granted by the fact that the two SPBs are not completely equal. Several proteins that play key roles in nuclear segregation indeed differently decorate the two SPBs, ensuring that the two structures do not behave in the same way and follow different segregation fates. These differential segregation routes are granted mainly through an initial inhibition of the microtubule nucleation from the newly synthesized SPB, which is retained by the mother, and through accumulation on the old SPB of dynein and the spindle positioning factor Kar9²¹¹⁻²¹³. Inhibition of the cell cycle by the SPOC is achieved through inactivation of the mitotic exit network (MEN), an essential pathway that drives cell from mitosis through cytokinesis. In response to spindle misorientation, the SPOC inhibits MEN apical GTPase Tem1 by stabilizing Bub2/Bfa1 complex, which acts as a GAP towards Tem1^{214,215}. In particular, the SPOC acts through two cortical proteins, Kin4 and Lte1, which are mainly restricted to the mother and daughter cortexes respectively²¹⁶⁻²¹⁸. Noteworthy, Lte1 recruitment to daughter cortex during mitosis relies on binding to active-Ras2²¹⁹⁻²²¹. As long as the spindle is not properly oriented, both SPBs are within the mother cell and subjected to Kin4 kinase activity that phosphorylates and prevents the Cdc5-mediated inactivation of the Bub2/Bfa1 GAP complex, thus preventing Tem1 triggering²²². Once a SPB passes through the bud neck, Lte1 interferes with Kin4 loading on it, allowing polo like kinase (PLK) Cdc5 to phosphorylate Bub2/Bfa1 and promote cell cycle progression triggering Tem1 activity^{223,224}. This in turn promotes a cascade of phosphorylation that ultimately results in unchaining of Cdc14 activity and completion of the cell cycle.

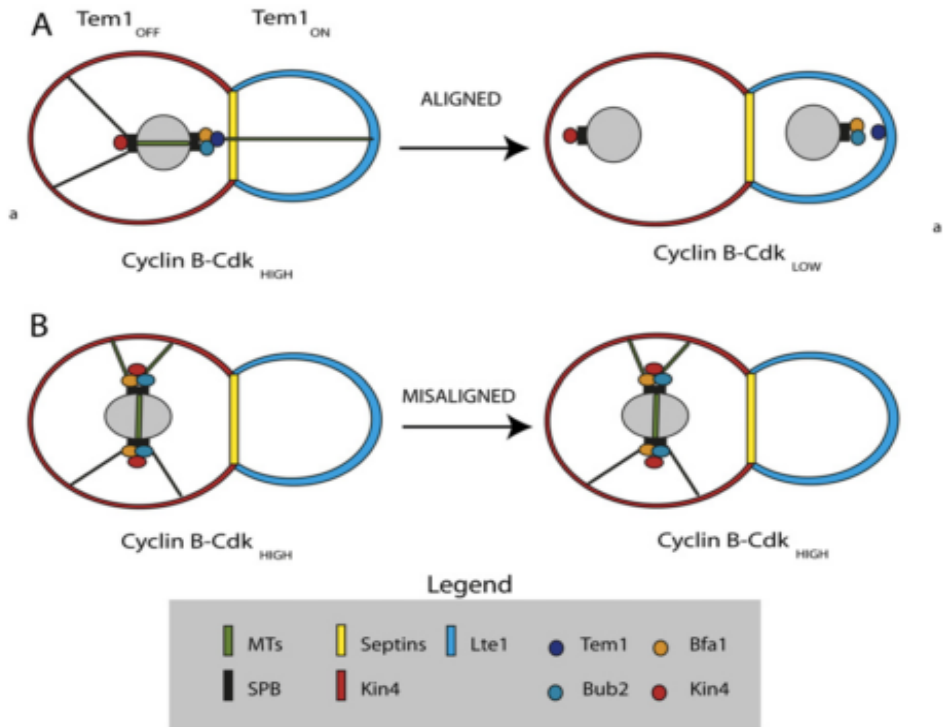


Figure I16. Schematic representation of the SPOC in presence of correct (figure A) and wrong spindle orientation (figure B)²²⁵

Non-canonical DNA Damage Response (DDR) activation

Preservation of the genetic material is fundamental for all living cells. Apart from threats deriving from missegregation, the fidelity of DNA transmission to daughter cells is jeopardized also by several other factors that can directly alter its sequence, leading to mutations. A plethora of factors ranging from physical agents to chemicals or to biological processes themselves can indeed impact on the DNA molecule and produce alterations. For this reason, complex mechanisms have evolved to preserve the integrity of the genetic information, collectively known as DNA Damage Response (DDR hereby) with the role to

trigger a transient cell cycle arrest and promote DNA repair, eventually driving cells through apoptosis in case of non-reparable damages.

The ample variety of chemically and structurally different DNA lesions are processed by different repair pathways. Whatever damage and repair pathway takes place, they all converge on a common mechanism to delay cell cycle progression, known as the DNA damage checkpoint, considerable as a phosphorylation-based signal transduction cascade that conveys the damage signal from the nucleus to several effectors involved in lesion processing and cell cycle control. The first event common to all injuries in checkpoint activation is the coverage of a stretch of ssDNA (generated by processing either ssDNA or dsDNA damage through the different repair mechanism) by the RPA complex²²⁶. After decoration of this ssDNA stretch by RPA, two protein complexes comprising DDR apical kinases are recruited to lesion sites with different mechanism. On one hand, ATR (Mec1 in budding yeast) is recruited along with the protein ATRIP mainly to ssDNA lesions, while ATM (Tel1 in *S.cerevisiae*) is recruited to processed double strand breaks. Once the apical kinases have been triggered, they transmit the signal through a phosphorylation cascade to their effectors, the first of which are Chk1 (ATR) and Chk2 (ATM). Due to its high relevance for carcinogenesis and pathogenicity, the DDR has been extensively studied to define the mechanism that drive pathology onsets and prevent them. DNA lesions are not the only stimulus that promotes DDR activation as a plethora of other conditions ranging from virus infection to chromatin structure alterations have been reported to trigger the DDR even in the absence of DNA lesions²²⁷. In a recent work, Kumar et al. showed that cells subjected to different kind of mechanical stress trigger an ATR-mediated activation of checkpoint factors on the nuclear lamina and reported evidences for this being a mechanism to protect perinuclear chromatin from potentially threatening torsional forces (fig.I17)²²⁸. This pathway may be highly relevant

for migrating or invading cells, where the cellular morphology is constantly manipulated and nuclear lamina has to disassemble to allow nucleus movement²²⁹. Consistent with this hypothesis, lamin A disruption has been reported to trigger DNA damage checkpoint and induce cell death in constricted cell migration²³⁰.

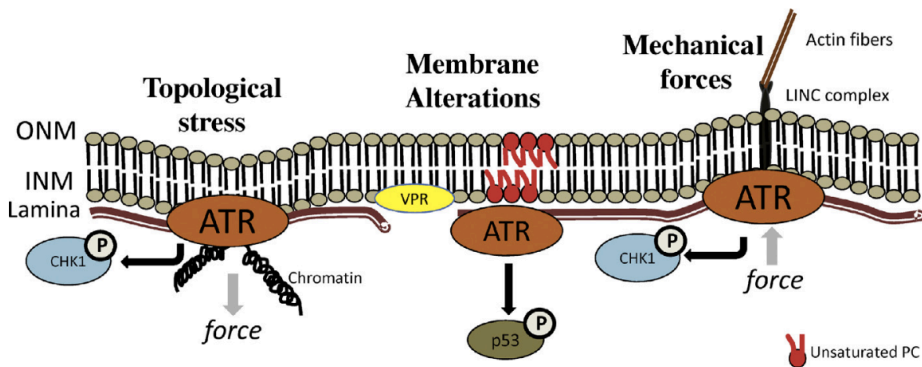


Figure I17. Mechanical stress on the nuclear envelope triggers ATR relocation²³¹

Haspin

The atypical serine/threonine kinase haspin (HAploid germ cell-Specific nuclear ProteIN kinase) was first identified in mouse testis cells in 1999 and later has been found in the genomes of all eukaryotes looked so far, including the minimal genome of the microsporidia *Encephalitozoon cuniculi*, suggesting an essential role in eukaryotic cells^{232–234}. The structure of haspin paralogues is variable from one organism to the other, but it has common features encompassing a kinase domain, several phosphorylation sites and a leucine zipper²³². In higher eukaryotes the haspin gene has some peculiar characteristics as it lacks introns, has some transposon-like features and it is located inside the intron of integrin alphaE²³⁵.

The best-known substrate of haspin is threonine 3 of the histone H3, which gets phosphorylated during mitosis and, together with phosphorylated histone H2A-

S121, recruits the Chromosome Passenger Complex (CPC)^{150,151,236}. Consistent with its role in CPC recruitment, impairment in haspin activity results in loosened chromatids, premature segregation, failure to align chromosomes on the metaphase plate and spindle-pole fragility^{234,237–239}.

Regulation of haspin involves both its localization and posttranslational modifications. On one hand, an autoinhibitory domain that in interphase folds onto the catalytic domain prevents unscheduled haspin activity²⁴⁰. This autoinhibition is relieved in a multi-step mechanism, the first of which is a Cdk1-mediated phosphorylation on residue T206 during mitosis and this modification primes haspin for Plk1 recognition and subsequent multiphosphorylation^{234,241,242}. Once phosphorylated, the autoinhibitory domain of haspin is displaced from the catalytic site, triggering haspin activity (fig.I18)²⁴¹. A second level of regulation exploits different localizations of haspin in the cell cycle: during interphase this protein is localized in the nucleus but not associated with chromosomes so it does not have access to its chromatinic substrates^{234,237}. Recruitment of haspin to chromatin is mediated by SUMOylated Topoisomerase II α and requires preliminary phosphorylation of haspin T206 phosphorylation by Cdk1^{243,244}.

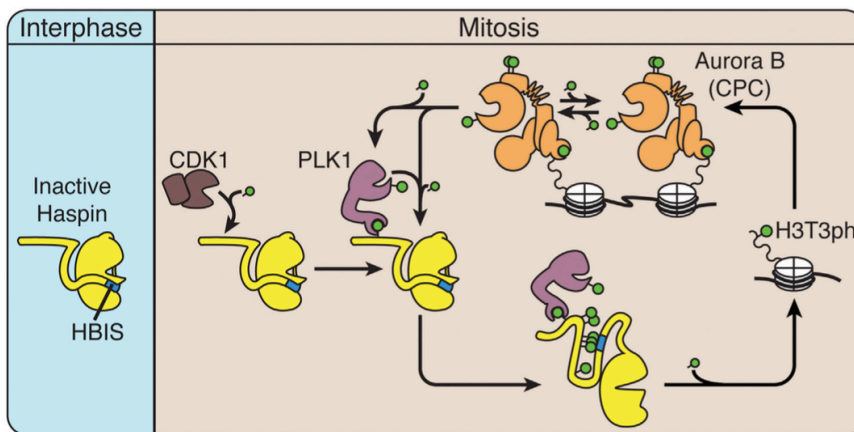


Figure I18. Regulation haspin activity in the cell cycle²⁴¹

Two haspin paralogues are coded by the budding yeast genome, Alk1 and Alk2²⁴⁵. Both proteins oscillate during the cell cycle, peaking at the first stages of mitosis²⁴⁶. Alk1 was first identified in our lab as an interactor of the DNA damage protein Ddc1 in a two-hybrid screening but no sensitivity of *alk1Δ*, *alk2Δ*, or *alk1Δalk2Δ* cells to genotoxic agents has been observed²⁴⁶.

Here, I show that haspin strongly impacts mitosis regulating actin and nuclear dynamics by modulating polarisome dispersion. In this scenario haspin exerts its role by affecting the distribution of Ras along the PM, which I show to modulate Cdc42 activation and polarity regulation. Moreover, preliminary results report an undisclosed role for haspin in regulation of a crosstalk between mechanosensing and DNA damage response.

AIM OF THE PROJECT

The main focus of this work is a deep comprehension on how haspin impinges on several aspects of the cell cycle.

Healthy cells in multicellular organisms are subjected to tight regulation mechanisms to prevent unscheduled growth and to sacrifice themselves when required for the wealth of the whole organism. Malignant transformation is a complex multistep process by which cells acquire characteristic traits that make them behave independently on extracellular signalling and non-responsive to apoptotic induction, conferring them growth advantages and the capability to spread in the organism outnumbering other tissues. This change in behaviour requires several alterations in cellular processes. One of the first steps often observed in carcinogenesis is the alteration of cellular polarity and of internal vesicle trafficking to favour cellular motility and proliferation. In this work I investigated the role of haspin in regulation of polarization and vesicle traffic using budding yeast as model.

Migrating and invading cells are subjected to strong mechanical stresses as they pass through a crowded environment and have to heavily manipulate their shape during this process. A limiting step in invasion is the passage of the nucleus, which has to be helped by Arp2/3-mediated rearrangements of F-actin and ruptures of the nuclear lamin^{229,230}. Treating cells with different mechanical stresses has been reported to trigger a non-canonical, ATR-mediated DDR²²⁸. How events acting on the cellular membrane are translated in DDR activation and what happens to migrating cells if this mechanism is perturbed is yet unknown. Given that our data in budding yeast and human cells indicate haspin to be a regulator of actin dynamics and haspin is known to be active in the nucleus, we hypothesized that it may couple sensing of mechanical stress to DDR activation²³⁷.

MAIN RESULTS

A first analysis performed in our lab reported budding yeast haspin paralogues Alk1 and Alk2 as mitotic proteins phosphorylated after DNA damage and undergoing cell cycle regulation but their role was not identified²⁴⁶.

Loss of haspin confers sensitivity to M-phase delays

Starting from Alk1 and Alk2 identification as mitotic proteins we tested haspin mutants sensitivity to microtubule-depolymerizing drugs as benomyl or nocodazole to assess the impact of M-phase arrests on such cells. Loss of haspin kinase activity results in a greatly reduced cell viability benomyl-containing plates, and direct analysis of the SAC (the surveillance mechanism which is triggered by microtubule depolymerization) demonstrated that this mechanism is proficient in haspin mutants, excluding a role in perceiving correct microtubule attachments. A more detailed analysis of the phenotypes of *alk1Δalk2Δ* cells monitoring spindle and nuclear dynamics after transient nocodazole treatment highlighted nuclear missegregation as the probable cause for *alk1Δalk2Δ* lethality following an M-phase delay, as this mutant undergoes cytokinesis with two separated nuclei in a single cell compartment following M-phase delays, resulting in binucleated daughters and anucleated mothers. A correct spindle elongation relies on a functional actin cytoskeleton and this prompted us to monitor the distribution of actin in haspin-lacking cells. Staining actin with fluorophore-conjugated phalloidin demonstrated that, indeed, loss of Alk1 and Alk2 causes, after an M-phase delay, a defect in actin distribution; while F-actin being is evenly distributed between mother and daughter cells in wild-type strains, it is strongly accumulated in the daughter in haspin mutants. Actin cable synthesis is regulated by formins and actin-nucleation promoting factors. Consistent with the observed defect in actin

cytoskeleton we found that Bnr1 (a formin) and Bud6 (an actin nucleation promoting factor) were mislocalized by loss of haspin and deletion of the corresponding genes could alleviate the phenotypes of haspin-lacking cells.

Dispersion of polarity clusters relies on a haspin-mediated active-Ras redistribution

These results prompted us to investigate the cause for the hyperpolarization observed in *alk1Δalk2Δ* cells. We then demonstrated that the lack of Bud6 from the bud neck and its concomitant accumulation at the bud tip were the actual cause for actin and nuclear misdistribution upon haspin loss. We found Bud6 misdistribution to be a consequence of defects in the master regulator of polarization in eukaryotes, the small GTPase Cdc42, and particularly of GTP-loaded Cdc42, which we report to be accumulated at the bud tip rather than being evenly diffused on the PM following haspin loss. The most obvious fact that would cause clustered accumulation of GTP-loaded GTPase is a similar distribution of its GEFs. Given that Cdc24 is the only GEF for Cdc42 in budding yeast, we then assessed its localization during M-phase in wild-type or *alk1Δalk2Δ* cells and found it being accumulated at the bud tip, consistent with Cdc42-GTP being polarized in this mutant. In contrast with literature evidences that ascribe Rsr1 the role to recruit Cdc24 to the PM, we found that in mitosis its loss had no impact on Cdc24 localization. On the other hand, we identified Ras as responsible for Cdc24 accumulation on the PM in this phase, confining Rsr1 role to G1. Finally, we demonstrated that active Ras and Ras are both polarized in haspin-lacking cells and that this is the cause for the M-phase sensitivity of this mutant.

Haspin impingement on vesicle and vacuolar dynamics

Delivery of Ras presumably relies on two distinct mechanisms based on secretory traffic and the palmitoyltransferase module Erf2/Erf4. However, the latter complex is also required for efficient membrane-binding of Ras, making it challenging to dissect its actual role in Ras accumulation on the PM. Our results show that Ras2 is hyperaccumulated at the bud tip upon haspin loss, so we reasoned that this may be due to defects in vesicle delivery. Indeed, we found that the v-SNARE Snc1 was accumulated at the bud tip rather than being uniformly distributed along the bud membrane in haspin mutants (supplementary informations, pag.142). Noteworthy, this phenotype was detectable also in conditions in which the actin cytoskeleton was unaffected by haspin loss, suggesting it was not just a consequences of published defects of *alk1Δalk2Δ* cells (supplementary informations, pag.143). The fact that Snc1 accumulation is not prevented by loss of haspin indicates that the secretory machinery per se is functional in such mutants, but rather vesicle are not evenly distributed to the cell periphery.

The PtdIns(3)*p* kinase Fab1 has been detected in our lab as a putative Alk2 interactor in a two-hybrid screening. We found several genetic interactions between haspin and Fab1 network overall suggesting that Alk1 and Alk2 could play a role in a correct assembly of Fab1 complex. Haspin is indeed involved in PtdIns balance, vacuole fragmentation and function and, though the exact mechanism of Fab1 regulation by haspin is still missing, it is likely that a strong interplay between the two proteins exists (supplementary informations, pag.144-145).

CONCLUSIONS AND FUTURE PROSPECTS

Carcinogenesis is driven by several alterations in cellular behavior that overall promote malignant phenotype and confers transformed cells the capability to spread in the whole organism. Among the processes more often altered in cancer cell is polarization, and hence understanding how polarity is established and maintained is of pivotal relevance in cancer research. This thesis deepens our comprehension on polarization as follows: it demonstrates that haspin oversees proper nuclear segregation along with polarity factors; it shows the outcome of failure in timely dispersion of polarisome; it describes a new role for Ras in Cdc42 regulation during mitosis and it characterizes haspin as a new regulator of vesicle delivery and PtdIns balance. Overall, the impact of our results on this research field can delineate new paths to elucidate the regulation of key proteins in carcinogenesis and cellular proliferation.

This work raises several new questions that will be assessed in the next future. The main challenge we will be facing is try to translate the results achieved in budding yeast to human cell lines. A success in this would define haspin as a new regulator of Ras, eventually defining new approaches in therapeutic Ras regulation and cancer treatment.

The way haspin acts on Fab1 complex is still unclear, and we will try to unveil the mechanistic details of this regulation. Our data raise the possibility that Alk1 and Alk2 may be act on Fab1, eventually to mediate complex assembly. Due to its high molecular weight, haspin-dependent protein modifications in Fab1 may remain elusive. To circumvent this, we will exploit the following experimental setups: on one hand, we know by personal communication that a TEV-cleavable Fab1 has been obtained and that this allele can be used to easily detect protein modifications; on the other hand, we could analyze Fab1 from wt and haspin-lacking cells by mass-spectrometry to identify differentially

modified residues that will be putative target for haspin kinase activity. The impact on haspin on Fab1 complex will be determined by Co-immunoprecipitating Fab1-Vac14-Fig4 in wt or *alk1Δalk2Δ*.

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PART II

PUBLISHED PAPER I

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

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Yeast Haspin Kinase Regulates Polarity Cues Necessary for Mitotic Spindle Positioning and Is Required to Tolerate Mitotic Arrest

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SUMMARY

Haspin is an atypical protein kinase that in several organisms phosphorylates histone H3Thr3 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 where the mitotic spindle is pulled. 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 suggests that this function of haspin is likely shared with other eukaryotes, in which haspin may regulate asymmetric cell division.

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 cell 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 the 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 recently been 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. 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 knockdown arrests cells in mitosis and prevents proper chromosome congression at the metaphase plate (Dai et al., 2005, 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 *Arabidopsis 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 H3Thr3 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

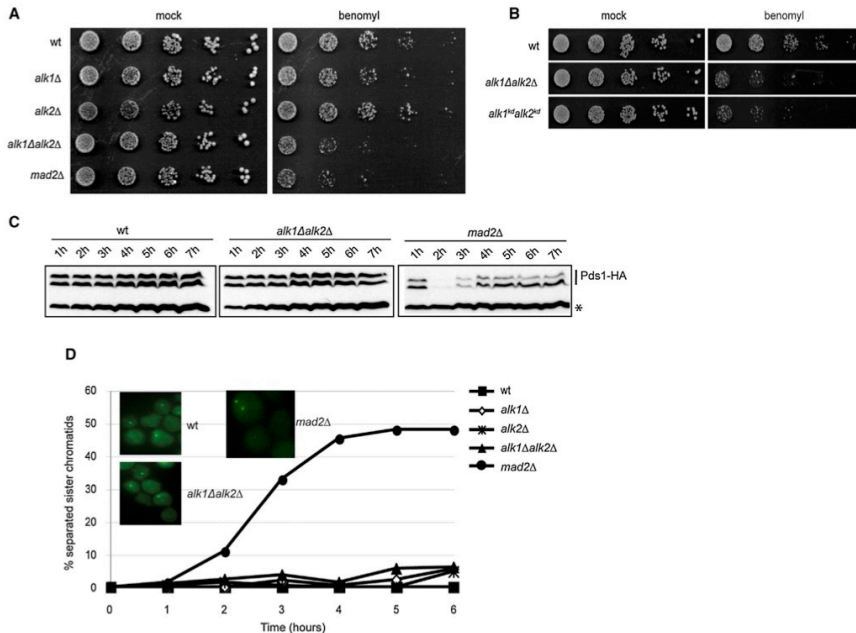


Figure 1. Yeast Haspin Deletions Cause Sensitivity to Microtubule-Depolymerizing Agents

(A) Serial dilutions of the indicated yeast strains grown on YPD (mock) or YPD + 8 μ g/ml benomyl plates. (B) Wild-type, *alk1Δalk2Δ*, and kinase-deficient double mutant (*alk1^{ts}alk2^{ts}*) treated as in (A). (C) Time course of Pds1 stability in cells arrested in α factor and released in 20 μ g/ml nocodazole-containing medium. The asterisk 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 insets present examples of fluorescence images showing normal sister chromatid cohesion (wild-type, *alk1Δalk2Δ*) or premature separation (*mad2Δ*). See also Figure S1.

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 explain 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.

RESULTS

Haspin-Defective Cells Are Sensitive to Microtubule-Depolymerizing Drugs

In human, *A. thaliana*, and *Schizosaccharomyces 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 *Saccharomyces cerevisiae*. Deletion of *ALK1* or *ALK2*, either singly 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 microtubule-depolymerizing drugs (i.e., benomyl or nocodazole) of cells deleted for the genes coding for the two haspin paralogs. As shown in Figure 1A, single *alk1Δ* or *alk2Δ* mutant cells show, respectively, mild or no sensitivity to benomyl. However, the viability of *alk1Δalk2Δ* double-mutant cells is heavily compromised in the presence of the drug, similar to what is observed in strains lacking the

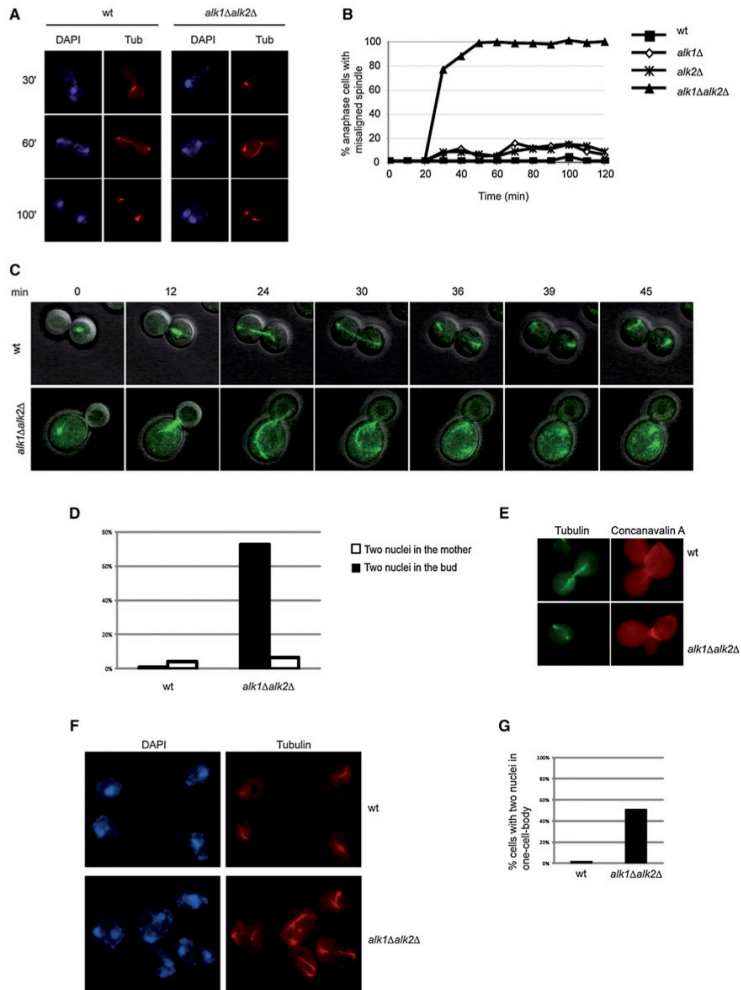


Figure 2. Lack of Haspin Impairs Mitotic Spindle Positioning after Mitotic Arrest

(A) Cells were arrested for 2.5 hr in nocodazole. Representative immunofluorescence pictures are presented, showing DNA (DAPI) and the mitotic spindle (Tub), taken at the indicated time points after nocodazole washout.

(B) Quantification of binucleated cells exhibiting incorrect mitosis after treatment as in (A).

(C) Kinetic analysis of spindle orientation after release from nocodazole arrest as in (A). Selected photograms from a time-lapse experiment (Movies S1 and S2) using strains expressing tubulin-GFP. The time point of each photogram is indicated.

(legend continued on next page)

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 liquid culture with nocodazole (Figure S1A available online). 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: depolymerizing the microtubule, activating the SAC, and 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 chromatid 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 and arrested in nocodazole-containing medium. As shown in Figure 1C, in *alk1Δalk2Δ* double-mutant cells, Pds1 is stable in nocodazole arrest, similar to what is observed in wild-type cells. On the contrary, in SAC-defective *mad2Δ* cells, Pds1 is rapidly degraded during 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 *alk1Δalk2Δ* 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 *alk1Δalk2Δ* 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 *alk1Δalk2Δ* mutant cells after release from a 2.5 hr nocodazole treatment (Figure 2; 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 cells. Strikingly, in *alk1Δalk2Δ* mutant cells, although 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 *alk1Δalk2Δ* anaphase cells contain two nuclei within a single cell body, whereas this aberrant population remains below 5% in wild-type and below 20% in *alk1Δ* or *alk2Δ* 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 *alk1Δalk2Δ* cells was due to alterations in spindle reassembly or to spindle positioning. After a transient 2.5 hr nocodazole treatment, we analyzed by time-lapse video microscopy cultures of wild-type and *alk1Δalk2Δ* 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 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 arresting *alk1Δalk2Δ* cells for 2.5 hr in nocodazole and removing the drug, the nucleus divides (Figure 2A), the misaligned spindle is timely disassembled (see Movies 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 *alk1Δalk2Δ* 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 easily be distinguished from round daughter cells. We released α factor 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 4',6-diamidino-2-phenylindole (DAPI). Fluorescence microscopy analysis of *alk1Δalk2Δ* cultures shows that in ~75% of cases, rounded binucleated and shmooed anucleated cells are detected, indicating that nuclear segregation occurs almost exclusively in the bud (Figure 2D). This result was further confirmed by staining mothers with concanavalin A before the arrest. Figure 2E shows

(D) Cells were treated with 10 μ g/ml α factor, and then released for 2.5 hr in medium containing 10 μ g/ml 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 *alk1Δalk2Δ* cells carrying Tub1-GFP after nocodazole washout. Cells were arrested as in (A).

(F) Wild-type and *alk1Δalk2Δ* cells, expressing *GAL1-CDC20*, were grown overnight in medium containing raffinose and galactose and presynchronized in G1 with α factor (5 μ g/ml). When 95% of the cells were unbudded, the cultures were washed and resuspended in medium containing glucose for 3.5 hr. 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).

See also Figure S2.

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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 (Movies S1 and S2) indicates that Alk1 and Alk2 are not involved in the 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, 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 reinduced to allow cells to progress through mitosis. As shown in Figures 2F and 2G, 60 min after anaphase entry, cells have finished mitosis: more than 50% of *alk1 Δ alk2 Δ* mutant 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. Whereas 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 Kar9 or 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* nor *dyn1 Δ* affects 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 redistributed 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 for 2.5 hr in nocodazole to induce metaphase arrest. Actin distribution was then analyzed at various times after nocodazole washout. As shown in Figure 3A, in nocodazole-arrested wild-type cells, actin cables are mostly undetectable and 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, consistent with the spindle misalignment phenotype and with the increased bud size observed when these mutants are arrested in mitosis. This result was also supported by staining mother cells with concanavalin A before mitotic arrest (Figure 3B). Confocal images confirmed the unbalanced actin distribution in haspin-mutated cells (Figure S3A).

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 nonpermissive temperature causes rapid disruption of the septin ring (Barral et al., 2000). *alk1 Δ alk2 Δ cdc12-6* cells were arrested with nocodazole at 25°C for 2.5 hr, shifted to 37°C for 45 min, and released in YPD at 37°C in the absence of the drug. Figures 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.

Because 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 fluorescence recovery after photobleaching (FRAP) analysis. Figures S3B and S3C show 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 Formin-Dependent Spindle Pulling Forces

Actin distribution is also controlled by formins, evolutionarily 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, whereas 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 for 2.5 hr; Figures 4A and 4B show that, after release from 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

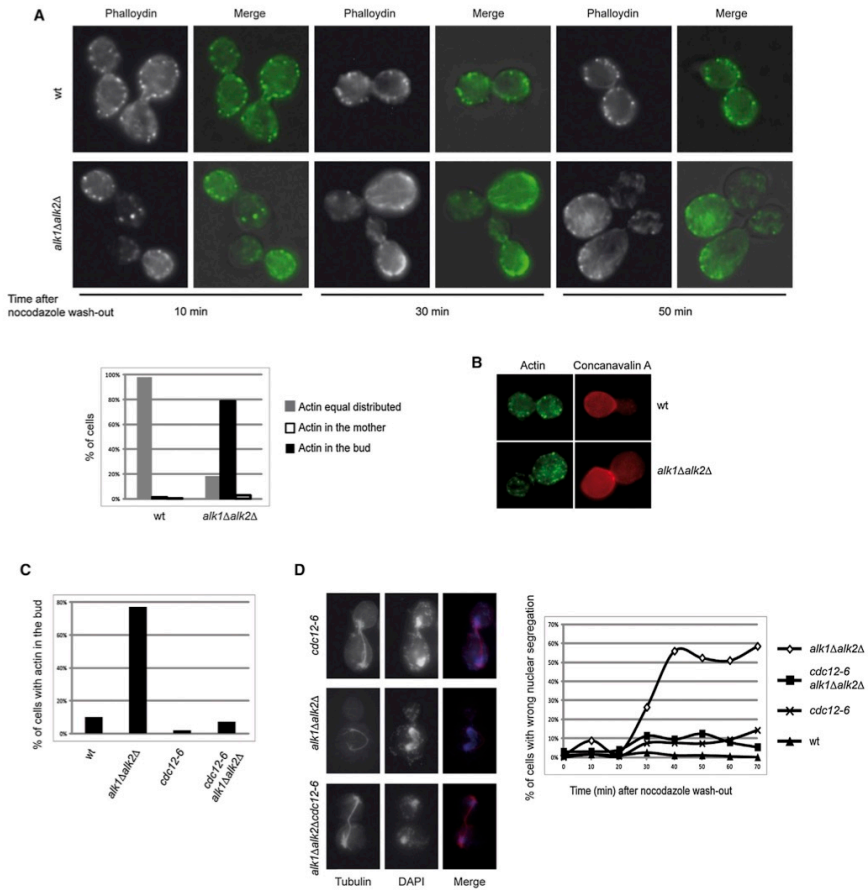


Figure 3. Haspin Is Essential for Proper Organization of Cell Polarity Factors in Mitosis
 (A) Actin distribution in the indicated strains was analyzed by fluorescence microscopy after phalloidin staining. Cells were arrested for 2.5 hr in nocodazole. Samples were collected at the indicated time points after nocodazole wash-out; selected images are shown with quantification of at least 200 cells/sample.
 (B) Concanavalin A-stained cells were analyzed for actin distribution as in (A). Selected images are presented.
 (C) Overnight cultures of wild-type, *cdc12-6*, *alk1Δalk2Δ*, and *alk2Δalk2Δcdc12-6* grown at permissive temperature were treated with nocodazole (10 μg/ml) for 2.5 hr, shifted to nonpermissive temperature for 45 min, and then released in fresh medium at nonpermissive temperature. Samples were collected at 20 min and stained with phalloidin. Quantification of at least 200 cells/sample is 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.
 See also Figure S3.

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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. Figures 4C and 4D show that combining *bnr1Δ* with *alk1Δalk2Δ* rescues part of the benomyl sensitivity (as seen by drop assay and by dose-response survival curve) and nuclear division defects caused by lack of haspin activity. Deletion of *BNR1*, 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., 2000). We thus investigated whether haspin depletion affected Bud6 localization. Cells expressing GFP-Bud6 were arrested in nocodazole for 2.5 hr 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 *alk1Δalk2Δ* cells, Bud6 is mislocalized: it is noticeably absent from the bud neck, and it is evidently accumulated within the bud (Figures 5A and 5B). The presence of Bud6 at the bud neck is important to establish and maintain symmetry in the spindle pole body 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 cable 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, whereas 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 imbalance 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 *alk1Δalk2Δ* released from 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 under 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 spindle pulling forces, keeping the

spindle pole bodies separated in the mother and daughter cells 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 the 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 (*mit2-3*) or its attachment to microtubules (*ndc80-1*). For this purpose, we combined *alk1Δalk2Δ* with *mit2-3* or with *ndc80-1*. At a nonpermissive temperature (37°C) both mutations are lethal, whereas at semipermissive temperatures (36°C for *mit2-3* 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) (Figure 6C). Figure 6B shows, by qualitative drop assays and by quantitative analysis, that in the absence of haspin both mutants lose viability even at semipermissive temperatures. 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 cuniculi* genome, which contains just 2,000 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, 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 for identifying 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 has 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 explain nocodazole-induced cell death. After release from nocodazole treatment, the kinetics of reassembly of the mitotic spindle is normal in *alk1Δalk2Δ* cells, but surprisingly, mutant cells exhibit a highly penetrant spindle positioning defect. Indeed, after a transient nocodazole treatment, cells lacking haspin position the mitotic

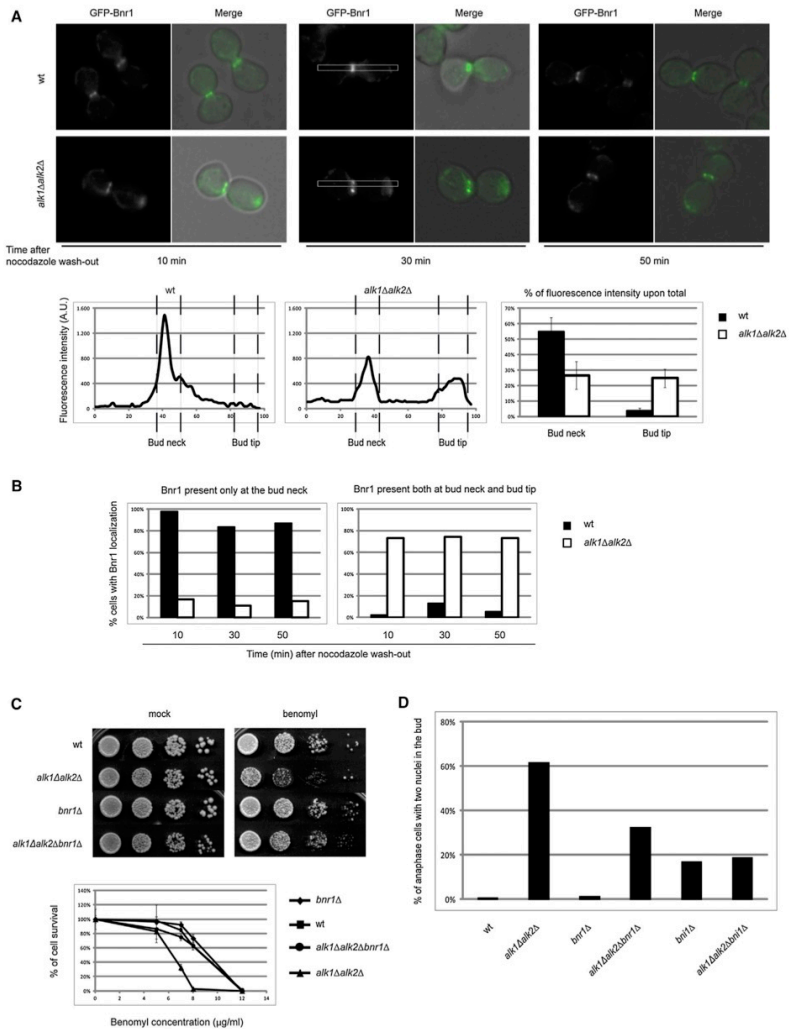


Figure 4. In *alk1Δalk2Δ* Cells, *Bnr1* Is Mislocalized and the Spindle Pulling Forces Are Unbalanced
(A) GFP-*Bnr1* localization was analyzed by fluorescence microscopy. Samples were collected at the indicated time points after 2.5 hr in nocodazole; representative images are shown. The fluorescence intensity along the mother-daughter cell axis (rectangular areas) has been quantified and is reported in arbitrary units in the line graphs. The locations corresponding to the bud neck and 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; error bars represent standard deviation.

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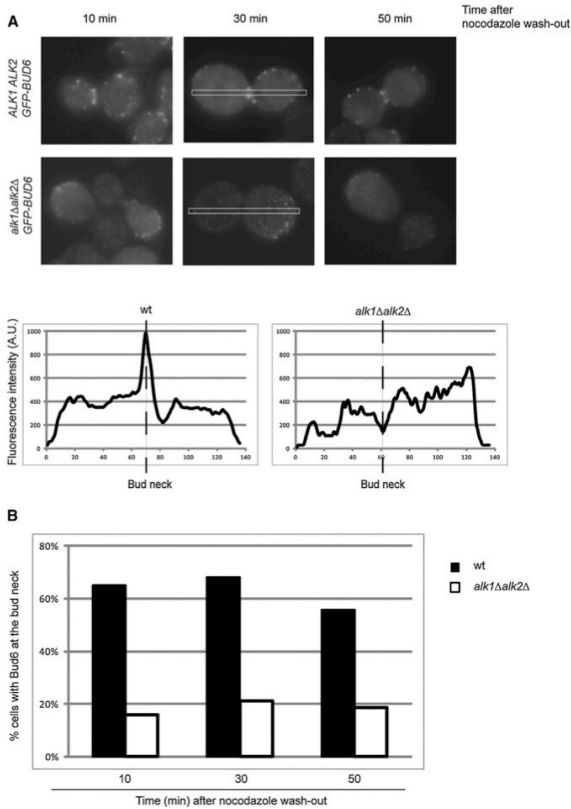


Figure 5. Localization of GFP-Bud6 in the Absence of Haspin

(A) Cells were arrested for 2.5 hr in nocodazole. GFP-Bud6 localization was analyzed by fluorescence microscopy at various time points after release from nocodazole. Representative images are shown. The fluorescence intensity along the mother-daughter cell axis (rectangular areas) has been quantified and is reported in arbitrary units in the line graphs. The locations corresponding to the bud neck are marked accordingly.

(B) Quantification of the experiment in (A). See also Figure S4.

spindle entirely within the bud, where it elongates and drives nuclear division, producing an anucleated mother and a binucleated daughter cell. To our knowledge, although 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 *alk1Δalk2Δ* cells. Our results indicate that yeast haspin has a role in allowing cells to tolerate the mitotic delay induced by SAC activation and reestablish 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, the polarisome complex, and formins. We show that, similar 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 have suggested physical and genetic interactions between polarization proteins and yeast haspin (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

(B) Quantification of the different categories observed in the experiment described in (A). At least 200 cells/sample were scored.

(C) Serial dilutions of exponentially growing cultures were plated on benomyl (8 μg/ml) and incubated at 23°C for 3 days. The quantitative analysis shown in the lower panel reports the percentage of cells that survive and form colonies on plates containing the indicated doses of benomyl; error bars represent standard deviation.

(D) Quantification of binucleated cells exhibiting incorrect mitosis 60 min after release from nocodazole treatment of the indicated yeast strains.

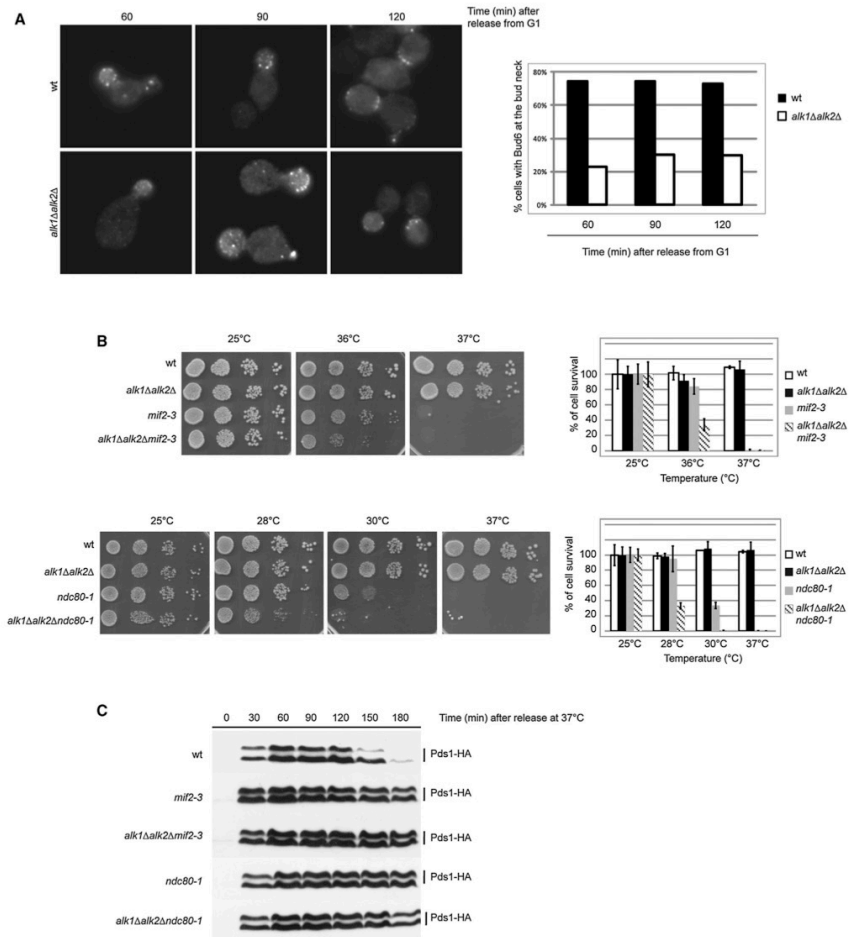


Figure 6. Haspin Function Is Required for Proper Localization of Polarity Cues during an Unperturbed Cell Cycle and Is Essential to Tolerate a SAC-Dependent Mitotic Arrest

(A) GFP-Bud6 localization in synchronized cultures was analyzed by fluorescence microscopy at the indicated time points after 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 dilutions of exponentially growing cultures of the indicated yeast strains were plated on YPD and incubated for 2 days at the indicated temperatures. The quantitative analysis shown on the right as bar graphs reports the percentage of cells surviving and forming colonies at the indicated temperatures; error bars represent standard deviation.

(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 analyzed by western blotting with 12CA5 (HA) antibodies.

See also Figure S5.

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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 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 imbalance 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 asymmetric 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 whether loss of haspin activity affects the correct execution of asymmetric cell division.

EXPERIMENTAL PROCEDURES

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 the endogenous promoter were kind gifts of M. Segal (Segal et al., 2000) and D. Pellman (Buttery et al., 2007). PCR-based genotyping was used to confirm gene disruption and tagging. Gene over-expression 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 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 μ g/ml), and released in the presence of nocodazole (10 μ g/ml). At different times, samples were collected to obtain total protein extracts that were resolved by SDS-PAGE and analyzed 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, samples taken at different time points were fixed with ethanol, and GFP was visualized by fluorescence microscopy with a Leica DMRA2 equipped with a 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 Alexa Fluor 488-conjugated phalloidin, actin was visualized by fluorescence microscopy as described above. Confocal images were acquired with a laser scanning confocal microscope (Leica TCS SP2). Images were processed by ImageJ.

Concanavalin A Staining

Cells were grown in YPD and synchronized with α factor (2 μ g/ml). After G1 arrest, cells were washed with PBS and resuspended in 125 μ l of Alexa Fluor 594-conjugated concanavalin A at a concentration of 40 μ g/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 Alexa Fluor 594-conjugated donkey anti-rat Ab (1:1,000), whereas DNA staining was performed with DAPI. For live-cell imaging, cells were adhered onto FluoroDishes (World Precision Instruments) and covered with synthetic dextrose medium. Cells were imaged at 30°C using a 63 \times immersion objective mounted on a Zeiss LSM 780 confocal microscope controlled by Zen 2010 software. Eight z stack images with an optical section spacing of 0.7 μ m were acquired every minute for 2 hr with a laser power of 0.4%. z stacks were max projected by ImageJ.

FRAP Analysis

FRAP experiments were performed as previously described (Dobbela et al., 2003) on a Zeiss LSM 510 confocal microscope. Briefly, logarithmically growing cells expressing GFP-Cdc12 were grown overnight in YEPD, treated with nocodazole (10 μ g/ml) for 150 min, and then resuspended in synthetic complete medium and spread on 2% agar pads. Half the septin ring was bleached with a sequence of 20–25 irradiations at 50% 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 two or three reference cells.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, one table, and two movies and can be found with this article online at <http://dx.doi.org/10.1016/j.devcel.2013.07.013>.

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PART III

MANUSCRIPT IN PREPARATION I

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

Ras Regulation by haspin triggers polarisome dispersion to allow M-phase delay tolerance

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Abstract

Cell polarization is of paramount importance for proliferation, differentiation and development. Its alterations are characteristics of carcinogenesis. How polarized factors are redistributed is not known. Here we identify haspin kinase as a factor critical in budding yeast for dispersion of the polarisome and link failure to disperse to nuclear segregation defects and cell lethality. This undescribed function of haspin relies on modulating the localization Ras. We describe a mitotic role for Ras and show that, physically interacting with Cdc24, it promotes activation of the Cdc42 GTPase on the bud plasma membrane. In *Saccharomyces cerevisiae* haspin is important for the regulation of mitotic spindle positioning and in the tolerance of mitotic delays, and our results explain the mechanism involved. These new findings shed light on critical factors that, controlling cell polarization and mitotic processes, may counteract tumorigenesis.

Introduction

Cells of almost all living organisms undergo a phase of polarization, in which material deposition and cell growth are directed towards specific areas of the cell periphery. Understanding the mechanisms overseeing this process is of pivotal importance since its deregulation can lead to severe diseases and is one of the first steps of malignant transformation in carcinogenesis¹.

A family of small proteins, Rho GTPases, oversees cellular polarity, with the protein Cdc42 playing a major role from budding yeast to human cells². In *S.cerevisiae*, this GTPase manipulates the cell shape by regulating processes ranging from vesicular trafficking to actin cytoskeleton dynamics, septin deposition and mating²⁻⁵. Cdc42 promotes budding during G1 from an otherwise round cell through its accumulation and activation at a polar cap and, after bud emergence, Cdc42 clustered activity at the bud tip directs growth of the daughter cell through manipulating of the actin cytoskeleton. At the end of mitosis, Cdc42 activity drops to allow cytokinesis⁶.

In budding yeast, the actin network is assembled thanks to two formins, Bnr1, which firmly associates to the bud neck, and Bni1, which accumulates at the bud tip⁷⁻¹¹. Bnr1 and Bni1 recruit Bud6, an actin nucleation promoting factor, at sites of actin cables synthesis^{12,13}. Bud6 enhances the actin nucleation activity of formins and regulates the early pathway of nuclear segregation¹²⁻¹⁴. In this scenario, Cdc42 is not required for actin cable assembly, but rather regulates their spatial organization during polarized growth, ensuring that a functional cytoskeleton is built, likely regulating formin distribution¹⁵. While the

establishment of polarization has been widely studied, the mechanisms underlying its dispersion and the consequences of its failure have not been investigated in detail.

The activity of Cdc42 is regulated by GTPase-Activating Proteins (GAPs), Guanin nucleotide Exchange Factors (GEFs) and Guanosine nucleotide Dissociation Inhibitors (GDIs). Budding yeast genome codes for a single GDI, Rdi1, while four GAPs (Rga1, Rga2, Bem2 and Bem3) are present in this organism^{16–19}. The only known GEF for Cdc42 in *S.cerevisiae* is the essential protein Cdc24, which orchestrates the accumulation of GTP-Cdc42 to differentially localized clusters during the cell cycle^{20,21}. In late G1, Cdc24 localizes at the presumptive bud-site and then, from S to M-phase it accumulates at sites of polarized growth; it is then sequestered into the nucleus during late M-phase until the next budding^{22,23}. Recruitment of Cdc24 at the PM in early stages of the cell cycle relies on its physical interaction with a Ras-family GTPase, Rsr1, and with the Bem1 scaffold protein. Clustered Cdc24 is responsible for the local activation of Cdc42 and is an absolute prerequisite for *S.cerevisiae* cells to bud²⁴. Deletion of both *RSR1* and *BEM1* is reported to be lethal²⁵. Interestingly, a small portion of *rsr1Δbem1Δ* cells is, to some extent, able to polarize and proliferate, indicating the existence of yet another player^{26–28}.

Work in different organisms suggested a physical interaction between Cdc24 and Ras, though the mechanistic details of this regulation and the impact of Ras on Cdc24 are lacking^{29,30}. Ras GTPases are ubiquitous in eukaryotic cells, where they play a fundamental role in cell cycle regulation and, noteworthy, Ras signalling is altered with a significant incidence in several types of human cancers³¹. In budding

yeast the main role of Ras paralogues, Ras1 and Ras2, is to regulate cell cycle commitment in G1 in response to external factors by activating PKA^{32,33}. Ras exerts this essential role upon accumulation on the plasma membrane (PM), achieved through a non-canonical vesicle delivery pathway dependent on mitochondria and class C VPS proteins^{34,35}. Activity of Ras in this organism is modulated by two GAPs (Ira1 and Ira2) and two GEFs, the essential Cdc25, and the dispensable Sdc25, which only takes part in Ras activation upon growth on poor media³⁶⁻⁴². Ras regulation likely occurs before PM delivery, as Cdc25, Ira1 and Ira2 are mainly localized to ER and mitochondria respectively³⁵. Beside its essential role in G1, some observations for Ras activity in mitosis have been reported in budding yeast and other organisms⁴³⁻⁴⁶. The atypical protein-kinase haspin is conserved in all eukaryotes, suggesting that it may play an important function in the cell cycle. Previous reports indicate that haspin is recruited at centromeric regions in a topoisomerase II dependent manner^{47,48}. Once there, haspin phosphorylates threonine 3 of histone H3 (H3-Thr3) and promotes efficient chromosome segregation through the recruitment of the Chromosome Passenger Complex (CPC), playing a critical role in ensuring a correct amphytelic attachment of microtubules to chromatids⁴⁹⁻⁵⁶. Recently H3-Thr3 phosphorylation has also been found to regulate asymmetrical histone inheritance in *Drosophila* male germline⁵⁷. In budding yeast, two haspin paralogues, Alk1 and Alk2, have been identified⁵⁸. We have recently shown that Alk1 and Alk2 play an essential role for tolerating a prolonged M-phase delay. Indeed, in cells where mitosis is delayed chemically or genetically, lack of haspin causes cell death due to the missegregation of both nuclei to the

daughter cell. This phenotype is accompanied by a strong hyper-accumulation of actin in the enlarged bud⁵⁹. We hypothesized that an altered regulation of polarization may be responsible for these phenotypes⁵⁹.

In this work, we analysed the involvement of *S.cerevisiae* haspin in polarization dispersion. Our findings confirm that mislocalization of Bud6, which in *alk1Δalk2Δ* cells is hyperpolarized to the bud tip and is missing from the bud neck, is the critical defect causing actin asymmetric distribution and uneven nuclear segregation. We show that yeast haspin ultimately regulates Cdc42, the master player of polarization. This function is exerted modulating the recruitment of Cdc24, the Cdc42 GEF, whose localization we demonstrate to be modulated by Ras. The possible evolutionary conservation of this new regulatory axis may help understand the unexplained effects on zygotic asymmetric cell division and embryonic patterning reported for *A.thaliana* haspin mutants.

Results

Haspin modulates Cdc42 activity in mitosis

The uneven distribution of actin and nuclei in haspin mutant yeast cells experiencing a mitotic delay may be a consequence of defective localization of polarity factors. In particular, Bud6 is hyperaccumulated at the bud tip and lost from the bud neck⁵⁹.

To verify whether the unbalanced distribution of Bud6 is responsible for the phenotypes observed in *alk1Δalk2Δ* cells, we retargeted Bud6 to the bud neck. A Bud6-Bni4 fusion protein forces localization of Bud6 at the bud neck also in the absence of haspin (Supplementary Figure 1). Figure 1 shows that Reinstating Bud6 at the bud neck is sufficient to recover a functional distribution of actin and a correct nuclear segregation in haspin mutant cells.

Failure to localize Bud6 at the bud neck may be due to failure in the local activation of Cdc42^{60,61}. To test whether loss of haspin causes defects in Cdc42 activity, we tried to rescue *alk1Δalk2Δ* cells by overexpressing *CDC42*. As shown in Figure 2a, induction of GAL-*CDC42* fails to restore a proper nuclear segregation in haspin mutants, raising the possibility that haspin may be required for a balanced distribution of active Cdc42. Consistently, overexpression of a constitutively active Cdc42-*G12V* suppresses the nuclear segregation defect of haspin-lacking cells, reducing it to the background level and restores the proper localization of Bud6 (Figure 2b)⁶². These results indicate that haspin activity is crucial to promote the correct distribution of active Cdc42.

A CRIB-TdTomato chimera that binds to GTP-loaded Cdc42 allows to specifically visualize the active form of Cdc42^{6,63,64}. Wt and *alk1Δalk2Δ* cells expressing this probe were subjected to an M-phase delay and analysed by fluorescence microscopy. GTP-loaded Cdc42 is homogenously distributed along the PM in the majority of control cells, with less than 40% cells exhibiting an increased signal of active Cdc42 at the bud tip (Figure 2c-e). In the absence of haspin, on the other hand, Cdc42-GTP is mostly detectable at the bud tips (85% cells; Figure 2c-e).

Importantly, no significant active Cdc42 is detected along the rest of the PM. We also measured the distance between the geometric centre of the cell (centroid) and the fluorescence centre of mass, a parameter that accounts for discrepancies from a uniform distribution of fluorescence. Consistently with the previous experiment, this value is significantly higher in *alk1Δalk2Δ* cells compared to wt controls (Supplementary Figure 2a).

Localization of polarity factors is a dynamic process and is followed by dispersion of the polarized proteins. Indeed, Cdc42 activity is known to be hyperpolarized at the bud tip in G1 and in mitosis is redistributed throughout the cell. Altogether, these results suggest that loss of haspin prevents redistribution of active Cdc42, which remains confined to the bud tip, leading to mislocalization of key polarity factors. The phenotype described above may thus result from a failure to disperse the polarity cap.

Haspin and Ras control Cdc24 recruitment to the PM in mitosis

Haspin may control proper localization of Cdc42 at activation sites, as suggested by Figure 2, or it may modulate Cdc42 activation at specific locations. In budding yeast, Cdc42 activity is regulated positively by a single, essential, GEF, Cdc24¹⁶⁻²¹; in particular, precise localization of Cdc24 is crucial to locally activate Cdc42. We thus investigated whether haspin may affect the localization of Cdc24.

We monitored GFP-Cdc24 in wt and *alk1Δalk2Δ* cells that were pre-synchronized in G1 and released in nocodazole-containing medium. In

wt cells, Cdc24 is distributed all over the PM, similarly to active Cdc42. In contrast, in absence of haspin, Cdc24 is undetectable except at the bud tip, explaining the elevated levels of active Cdc42 at the same location (Figure 3a-c and Supplementary Figure 3a). This suggests that in the absence of haspin, a massive accumulation of Cdc24 at the bud tip is responsible for altered distribution of Cdc42-GTP.

We then investigated what regulates Cdc24 distribution at the cell membrane. In G1, initial accumulation of Cdc24 at the presumptive bud site is promoted by Rsr1²⁶. *RSR1* deletion does not rescue Cdc24 mislocalization in haspin mutants (Supplementary Figure 3b), suggesting that an Rsr1-independent pathway controls Cdc24 localization in mitosis. Another factor that regulates Cdc24 recruitment at the beginning of the cell cycle is Bem1. To test whether the defective localization of Cdc24 upon haspin loss was due to alterations in Bem1 distribution, we monitored Bem1-GFP localization in wt or haspin-lacking cells subjected to a M-phase delay. As shown in Supplementary Figure 3c, loss of Alk1 and Alk2 caused a slight increase in the percentage of cells with Bem1 accumulation at the bud tip, yet the protein was still polarized also in control cells and the observed distribution pattern was not similar to that of Cdc24, suggesting other factors could be responsible for localization of the GEF during mitosis.

In the fungus *C.neoformans* and in *S.pombe*, Cdc24 was reported to physically interact with Ras, although the functional significance has not been determined^{29,30}. Budding yeast genome encodes two Ras paralogues, Ras1 and Ras2. Viable double mutant cells can be obtained by removing Bcy1, the inhibitory subunit of PKA.

We investigated the possible involvement of Ras in modulating Cdc24 localization in mitotic cells by deleting *RAS1* and *RAS2* in wt and *alk1Δalk2Δ* cells carrying a *bcy1Δ* allele, and analysing Cdc24 in nocodazole-arrested cultures.

In wt cells, removal of Ras1 and Ras2 significantly decreases the normalized Cdc24 fluorescence intensity ratio between PM and cytoplasm (Figure 3e, 1=homogeneous distribution between membrane and cytoplasm), while the fraction of cells showing Cdc24 at the bud tip slightly increased, suggesting that Ras may be required for Cdc24 recruitment to the PM during mitosis. In haspin lacking cells, loss of Ras greatly reduced the recruitment of Cdc24 to the bud tip (Figure 3d and Supplementary Figure 3d).

Moreover, we found that Ras was responsible for the defective localization of Cdc24 in haspin-lacking cells as its loss lowered GEF accumulation at the bud tip (Figure 3d and Supplementary Figure 3c). Finally, we noticed a slight yet significant increase in the percentage of cells showing polarized Cdc24 in Ras mutants compared to wt cells. We propose that this is caused by removal of mitotic-specific Cdc24 recruitment mechanism that leaves the GEF able to partially interact with players that regulate its distribution in earlier phases of the cell cycle (e.g.: Rsr1) and that are eventually still confined to the bud tip in M-phase.

To determine whether a physical interaction with Ras may mediate Cdc24 relocalization, we adopted a BiFC (Bimolecular Fluorescence Complementation) approach: the association of two proteins, fused to Venus^N and Venus^C respectively, is revealed by a fluorescent signal that is produced only if the chimeric proteins interact⁶⁵. We measured by flow

cytometry the fluorescent signal in logarithmically growing cells expressing endogenous Cdc24 tagged with Venus^N and Ras2 tagged with Venus^C. The presence of both chimeras reconstitutes a functional Venus, causing a shift in the fluorescence intensity profile, with respect to the non-tagged control. Importantly, the signal is reduced by competing with overproduced untagged Ras2, proving the specificity of the assay (Figure 4a). The interaction is also detectable as a fluorescent signal by microscopy and even in this case overproduction of untagged Ras2 suppresses the fluorescent signal (data not shown). To characterize the Cdc24-Ras2 interaction we performed a spatio-temporal analysis by fluorescence microscopy. Cells were classified, according to their bud size, in unbudded (G1), small budded (bud size less than 1/3 of the mother size; S phase) and large budded (bud size greater than 1/3 of the mother size; G2-M). Cdc24-Ras2 interaction is detectable in all cell cycle stages, but its cellular distribution varies greatly (Figure 4c). In most unbudded cells (87%), the signal is distributed all over the PM, and a small fraction (6%) of cells exhibit a preferential accumulation in discrete clusters. Small budded cells accumulate Cdc24-Ras2 complex on the PM of the mother cell (80%), while the complex is almost always absent from daughter membranes and few buds exhibit accumulation of the complex at their tips (13%). Large-budded cells show a preferential accumulation of Cdc24-Ras2 complex either at the bud tip (34%) or throughout the PM in both mother and daughter cells (60%). Intriguingly, when we analysed the impact of haspin on modulating the Cdc24-Ras2 complex during a mitotic arrest (Figure 4b), we observed that while the complex is evenly distributed on the PM in wt cells, in *alk1Δalk2Δ* cells Cdc24-Ras2 interaction is limited

to the bud tip, suggesting that haspin regulates Ras-dependent Cdc24 localization.

Recruitment of Cdc24 at the incipient bud site (G1) and later at the tip of the developing bud (early S phase) has been shown to rely on Rsr1⁶⁶. The mechanism responsible for the clustering of active Cdc42 at later cell cycle stages were not described. Our results identify Ras as a critical factor, which directly recruits Cdc24 to the PM. Cdc24-Ras2 interaction is particularly relevant to promote a correct redistribution of active Cdc42 in mitosis, where other factors known to regulate Cdc24 localization in earlier cell-cycle stages are not active.

Haspin regulates GTP-Ras dynamics

Previous data from other organisms suggest that the GTP-bound form of Ras is involved in the interaction with Cdc24. We have shown above that haspin is required to delocalize Cdc24-Ras2 from the bud tip in mitosis. These observations may be explained if haspin regulated the distribution of active Ras. To check this hypothesis, we exploited a GFP RBD (Ras Binding Domain from human Raf1) fusion, which specifically binds active Ras and has been used to monitor localization of GTP-Ras in the cell⁶⁷. After a mitotic delay, in cells lacking haspin active Ras is strongly hyperpolarized towards the bud tip, while in control cells GTP-Ras is distributed throughout the plasma membrane of both mother and daughter cells (Figure 5a-c). This finding is confirmed by measuring the centre of mass-centroid distance, which is higher in haspin-lacking cells (Supplementary Figure 5a). Noteworthy, overexpression of either wt or

hyperactive Cdc42 does not rescue this altered localization, indicating that Ras acts upstream of Cdc42 (Supplementary Figure 5b).

We previously reported that in *alk1Δalk2Δ* cells some polarity factors are mislocalized also in unperturbed cycling cells⁵⁹. To assess whether active Ras localization is altered even in these conditions, we synchronized wt and *alk1Δalk2Δ* cells in G1 and followed throughout the cell cycle, scoring the percentage of cells with polarized GFP-RBD signal. We found that cells enter the cell cycle with no evident clusters of Ras-GTP. Subsequently, 60-70 minutes after the release (approximately 10' after completing S-phase), a high percentage of cells polarizes active-Ras both in wt and haspin-lacking strains. However, this accumulation is transient in control cells, almost completely disappearing at 90' after the G1 release, when Ras-GTP acquires a more uniform cortical distribution. On the other hand, loss of haspin results in more pronounced and persistent polarized Ras-GTP clusters, which are redistributed only 110' after release from G1, at cytokinesis (Figure 5a-d and Supplementary Figure 5c). This result demonstrates that haspin is a critical factor that ensures a proper distribution of Ras activity in the cell. Failure to redistribute Ras-GTP, Cdc24, Cdc42-GTP, Bud6 before metaphase completion leads to nuclear missegregation and cell lethality when anaphase onset is delayed.

Ras activity is modulated by two GAPs, Ira1 and Ira2, and two GEFs, Cdc25 and Sdc25³⁶⁻⁴². Since Sdc25 is active only in particular nutrient conditions, we investigated the possibility that mislocalization of Cdc25 may be responsible for the altered distribution of active-Ras in haspin-

defective cells⁴². As shown in Supplementary Figure 6a, we found no differences in Cdc25 distribution in wt or *alk1Δalk2Δ* cells during an M-phase delay. If loss of haspin led to impairments in the GAPs, then deletion of *IRA1* and *IRA2* may restore wt phenotypes. Supplementary Figure 6b shows that removal of Ras GAPs has only a minor attenuating effect on the nuclear and actin defects of haspin-lacking cells, suggesting that these proteins do not play a significant role in establishment of such phenotype. Since haspin does not modulate the positive and negative regulators of Ras, it may control the proper localization of the global pool of Ras protein. Analysis of localization of GFP-Ras2 during a nocodazole treatment confirmed that deletion of *ALK1* and *ALK2* caused the accumulation of Ras2 protein at the bud tip, while this protein is distributed homogeneously on the PM in wt cells (Figure 6a-b and Supplementary Figure 6d). Together, these results indicate that in budding yeast haspin redistributes Ras rather than controlling its activation.

Ras oversees polarisome dispersion to ensure correct nuclear segregation in budding yeast

So far, our results are consistent with Ras playing a major role in response to mitotic delays in budding yeast. We then wondered how Ras-lacking cells could cope with such stresses. To test this, cells were treated with nocodazole for 3 hours and released in fresh medium taking samples to monitor actin (0' after the release) or nuclei (60' after the release). As expected from previous experiments, we observed that deletion of *RAS1* and *RAS2* in *alk1Δalk2Δ* strains was sufficient to

restore nuclear missegregation and actin misdistribution to that of a *ras1Δras2Δ* background (Figure 7a-b). Moreover, consistently with the partial Cdc24 accumulation at the bud tip in *ras1Δras2Δbcy1Δ* cells, loss of Ras caused a significant increase in the percentage of actin misdistribution and binucleated cells (Figure 7a-b), definitely ascribing Ras proteins an undescribed role in polarity and nuclear segregation monitoring. Noteworthy, both actin accumulation and nuclear missegregation occurred preferentially in daughter *ras1Δras2Δbcy1Δ* cells (Figure 7c-d).

Discussion

Alterations in cellular polarity lead to various diseases and carcinogenesis, making understanding the basis for polarity regulation a key challenge in research. Budding yeast *Saccharomyces cerevisiae* has proven to be an invaluable tool to dissect the way polarity onset occurs at the level of the small GTPase Cdc42 and its positive (GEF, namely Cdc24) or negative (GAPs and GDIs) regulators. Studies in this organism, indeed, provided a wealth of information on how polarization is established and maintained to allow proper cell growth. On the other hand, however, we still lack a complete picture of how cells cope with polarity dispersion and what are the outcomes of a failure in such process. Previously, we provided a first insight on the consequences of a prolonged polarization on budding yeast cells. Indeed, we observed that *alk1Δalk2Δ* cells, in which haspin-paralogues are lacking, are unable to redistribute polarity factors from the bud tip after an M-phase

delay, and this causes actin accumulation, nuclear missegregation and ultimately cell death⁵⁹. Here we have shed further light on the mechanisms underlying polarisome dispersion, and results presented clearly prove that timely relocalization of polarity proteins is a fundamental event in the cell cycle.

One of the observations from our previous work was that the actin nucleation-promoting factor Bud6, which is also involved in nuclear segregation mechanisms, was mislocalized in lack of haspin, and ascribed this as the leading cause for actin and nuclear defects in such cells. In this work, we demonstrate that these phenotypes are actually due to the failure to recruit Bud6 to the bud neck and its unbalanced accumulation at the bud tip. Indeed, we managed to restore a mostly wt phenotype in haspin mutants forcing Bud6 to localize at the bud neck through a Bud6-Bni4 fusion (Figure 1 and Supplementary Figure 1). Though not surprising, this observation provides a first mechanistic insights on how faithful nuclear segregation and symmetric redistribution of actin are achieved.

One of the peculiar outcomes of haspin loss is the build up of a misorganized actin cytoskeleton, that we demonstrated to be caused by unbalanced Bud6 distribution. Bud6 is an effector of the small GTPase Cdc42, the master regulator of polarization in budding yeast and impairments in Cdc42 result in the building of non-properly organised actin networks¹⁵. Cdc42 has also been shown to regulate both actin and nuclear segregation in human cells, making it an appealing candidate for a haspin-dependent regulation⁶⁸. We reasoned that if haspin-lacking cells were defective in Cdc42, overexpression of the GTPase could rescue their phenotypes. This approach did not work (Figure 2a-b).

However, overexpression of a constitutively active Cdc42 led to a complete rescue of nuclear segregation and actin distribution defects in haspin-lacking cells. Consistent with the fact that restoring Bud6 at the bud neck is sufficient to relieve defects of *alk1Δalk2Δ* cells hyperactive Cdc42 also restored Bud6 at the mother/daughter interface. This strongly suggested that *alk1Δalk2Δ* cells could be defective in activation of the GTPase rather than on its localization and hence prompted us to evaluate it in budding yeast cells. We proposed then that Alk1 and Alk2 are important in mitosis for the proper localization and activation of Cdc42. Indeed, in the absence of haspin GTP-Cdc42 was accumulated at the bud tip compared to wt control cells (Figure 2c-e and Supplementary Figure 2).

We hence hypothesized that haspin may be relevant to modulate local Cdc42 activation in mitotic cells reasoning that this would be consistent with the fact that constitutively active Cdc42 suppressed the *alk1Δalk2Δ* phenotypes but its wt counterpart did not. We then analysed the distribution of Cdc24, the GEF responsible for activating Cdc42, as its mislocalization could result in the hyperaccumulation of active Cdc42 only at the bud tip, preventing polarisome dispersion. In mitotic wt cells, Cdc24 is dispersed all over the cell membrane, reflecting the homogenous distribution of GTP-Cdc42 and presumably promoting polarisome dispersion. Conversely, in the absence of haspin Cdc24 is mostly found at the bud tip, explaining why GTP-Cdc42 accumulates in the same region (Figure 3a-c and Supplementary Figure 3a).

At the beginning of the cell cycle Cdc24 localization at the incipient bud site is established through the interaction with the Ras-family protein Rsr1 and the scaffold protein Bem1, however *RSR1* deletion had no

impact on Cdc24 localization in mitotic cells, while localization of Bem1 was different from the distribution of Cdc24 (Supplementary Figure 3b-c). Budding yeast contains two Ras paralogues, and in other fungi a functional and physical interaction between Ras and Cdc24 has been proposed, although the relevance of such interplay has not been assessed^{29,30}. When we deleted Ras-coding genes in *alk1Δ alk2Δ* cells, we found that Cdc24 accumulation at the bud tip was reversed and the defect due to loss of haspin were rescued (Figure 3d and Supplementary Figure 3d). Moreover, the overall abundance of Cdc24 at the plasma membrane was noticeably reduced by removal of Ras proteins, also in *ALK1 ALK2* M-phase arrested cells (Figure 3e), demonstrating that Ras recruits Cdc24 at the plasma membrane in M-phase to promote Cdc42 activity.

This last finding together with the notion in literature that Cdc24 binds Ras in other organisms, suggested that Cdc24 localization could rely on a direct physical interaction with Ras. We demonstrated this hypothesis with a BiFC approach, first in diploid strains and then monitoring the distribution of Cdc24-Ras2 complex in aploid cells. Our results show that the two proteins interact along all the cell-cycle but the localization of the complex varies during cell growth (Figure 4 and Supplementary Figure 4). We found the Cdc24/Ras complex to be constantly bound to the PM of virtually all budded and unbudded cells analysed. Only a small fraction of small-budded cells shows accumulation of Cdc24-Ras2 at the bud tip; suggesting that Ras2 is not physiologically required for initial recruitment of the GEF early in the cell-cycle. The presence of small-budded cells that however showed a daughterly accumulation of the complex however suggests that Ras may account for the reported

capability of some *rsr1Δbem1Δ* strains to successfully polarize Cdc24²⁶⁻²⁸. Later in the cell-cycle, when daughter approaches mother size, the incidence of polarized interaction increases, with the complex being then likely displaced from the polarity cap to favour its uniform distribution along both mother and daughter PM, as also suggested by our result in nocodazole.

This pattern of interaction together with established notions on how Cdc24 gets accumulated at the bud tip supports a bipartite model for Cdc24 recruitment during the cell cycle. In early stages, Rsr1 and Bem1 cooperatively promote accumulation of the GEF at the bud tip while Ras is mostly dispensable for this process. As cell-cycle proceeds, there likely is a change in Cdc24 binding partners, and the protein is likely detached from Rsr1 to promote its binding to GTP-Ras2. The ultimate outcome of this process is the redistribution of Cdc24, followed hence by GTP-Cdc42, from the bud tip to the whole PM. This is not the first report of proteins relocating from the bud tip to the PM by physical interaction with active Ras. Works by Yoshida et al. and Geymonat et al. indeed showed that Lte1, which initially accumulates at the bud tip, is recruited during mitosis to the PM following a series of phosphorylation events that promote its binding to GTP-Ras and we propose that a similar mechanism may exist also for Cdc24 and eventually, given the interplay between Ras and Cdc24 paralogues, being conserved in higher eukaryotes^{44,69}.

We demonstrated that Haspin loss causes Cdc24 accumulation at the bud tip; this, together with the notion that Cdc24/Ras complex is hyperpolarized in these cells, the fact that loss of Ras prevents Cdc24 recruitment at the PM and that Cdc24-Ras interaction is specific towards

GTP-Ras suggested that active Ras could be hyperpolarized too in *alk1Δalk2Δ* cells. We assessed this possibility monitoring the localization of GTP-Ras using a GFP-RBD probe, by which we determined that lack of haspin had a profound impact on the distribution of active-Ras in M-phase arrested cells (Figure 5a-c and Supplementary Figure 5a), which could not be suppressed by hyperactive Cdc42, supporting the notion that Ras acts upstream of Cdc42 in this haspin-regulated pathway (Supplementary Figure 5b). Interestingly, during an unperturbed cell-cycle, recruitment of GTP-Ras at the bud tip was detected both in wt and in *alk1Δalk2Δ*, making it a physiological event in the cell cycle. However, only in haspin mutants a prolonged maintenance of the bud-tip cluster of GTP-Ras was observed (Figure 5a,d and Supplementary Figure 5c). This result is particularly interesting as it provides a timing mechanism for polarisome dispersion during M-phase: if cells progress efficiently through mitosis, the temporary GTP-Ras accumulation at the bud tip does not lead to any negative effect. On the other hand, if cells experience a mitotic delay, the cluster of active Ras at the bud tip has to be dispersed through the action of haspin. Failure to remove this cluster triggers a cascade of perturbations in protein localization that ultimately results in nuclear missegregation and cell death.

These results collectively demonstrated that GTP-Ras dynamics manipulate the localization of Cdc24 and hence, by modulating active Cdc42 distribution, impact on key processes of budding yeast mitosis ranging from polarization to nuclear segregation. One unsolved question was then how haspin manipulates distribution of GTP-Ras. An obvious mechanism may involve its GEF and GAPs. Results reported in Supplementary Figure 6a-b demonstrate that this is not how haspin

controls active Ras. Instead, Alk1 and Alk2 regulate the localization of the general pool of Ras, as in the absence of haspin, we found a strong accumulation of Ras2 at the bud tip (Figure 6a-b and Supplementary Figure 6d).

This newly unveiled function of active-Ras raised the question of what happens to cells lacking Ras when they experience mitotic delays. Interestingly, though it causes a slight yet reproducible increase in actin and nuclear segregation defects, loss of Ras is sufficient to alleviate the phenotypes of haspin-lacking cells to a *ras1Δras2Δbcy1Δ* background (Figure 7a-d).

Our results demonstrate that haspin is responsible for the dispersion of polarity factors from the bud tip, and this process is required to tolerate M-phase delays. In particular, haspin controls localization of GTP-loaded Ras that in turn is responsible for directly recruiting Cdc24 along the PM to allow redistribution of GTP-Cdc42, demonstrating that Ras proteins play a critical role during mitosis, as schematically represented in Figure 8. This work provides, to the best of our knowledge, the first mechanistic insights on how depolarization process is promoted. How haspin regulates Ras localization is still unknown. However, we can speculate that the most likely events leading to the observed hyperpolarization could be an augmented delivery of Ras-containing vesicle to the bud tip, an excessive recycling of Ras from the PM or either an altered diffusion rate of Ras along the membrane. This aspect of haspin function will need further experimental analysis.

Methods

Yeast Strains and Plasmids

All strains used in this study are isogenic to W303, and are listed in Table1. Standard conditions for yeast cell cultures have been previously described⁷⁰. Standard molecular genetics techniques were used to construct plasmids and strains. The centromeric plasmids containing, *GFP-3RBD* and *CDC25-eGFP* were kind gifts of Dr. E.Martegani⁶⁷. *GFP-BUD6* and *CDC24-eGFP* bearing strains were obtained transforming cells with pRB2190 and pYS37 respectively^{71,72}. Bem1-GFP and CRIB-TdTomato were kindly provided by Dr. D.Lew⁶⁴. PCR-based genotyping was used to confirm gene disruption and tagging. Gene overexpression or repression with the inducible *GAL1* promoter was achieved by adding 2% galactose or 2% glucose respectively to raffinose-containing medium.

Strains used in this work

All the strains used are isogenic to W303.

NAME	RELEVANT GENOTYPE	SOURCE
W303	<i>ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 MATa/α</i>	R.Rothstein
k699	<i>ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 MATa</i>	K.Nasmyth

yAN33	<i>alk1::NATr alk2::HIS3 MATa</i>	This work
yRQ301	<i>CRIB-TdTomato-KANr MATa</i>	This work
yRQ302	<i>alk1::NATr alk2::HIS3 CRIB-TdTomato-KANr MATa</i>	This work
yRQ315	<i>[GFP-BUD6][GAL-CDC42] MATa</i>	This work
yRQ316	<i>alk1::NATr alk2::HIS3[GFP-BUD6][GAL-CDC42] MATa</i>	This work
yRQ317	<i>[GFP-BUD6][GAL-CDC42-G12V] MATa</i>	This work
yRQ318	<i>alk1::NATr alk2::HIS3 [GFP-BUD6][GAL-CDC42-G12V] MATa</i>	This work
yRQ255	<i>[GFP-BUD6-Bni4ter] MATa</i>	This work
yRQ256	<i>alk1::NATr alk2::HIS3 [GFP-BUD6-Bni4ter] MATa</i>	This work
yRQ214	<i>[GFP-BUD6-BNI4-BNI4ter] MATa</i>	This work
yRQ215	<i>alk1::NATr alk2::HIS3 [GFP-BUD6-BNI4-BNI4-ter] MATa</i>	This work
yRQ100	<i>[CDC24-GFP] MATa</i>	This work
yRQ101	<i>alk1::NATr alk2::HIS3 [CDC24-GFP] MATa</i>	This work
yRQ342	<i>rsr1::KANr [CDC24-GFP] MATa</i>	This work
yRQ343	<i>alk1::NATr alk2::HIS3 rsr1::KANr [CDC24-GFP] MATa</i>	This work
yRQ299	<i>BEM1-GFP-LEU2 MATa</i>	This work
yRQ300	<i>alk1::NATr alk2::HIS3 BEM1-GFP-LEU2 MATa</i>	This work
yRQ366	<i>bcy1::KANr [CDC24-GFP] MATa</i>	This work
yRQ367	<i>alk1::NATr alk2::HIS3 bcy1::KANr [CDC24-GFP] MATa</i>	This work

yRQ368	<i>ras1::TRP1 ras2::HPHr bcy1::KANr [CDC24-GFP] MATα</i>	This work
yRQ369	<i>alk1::NATr alk2::HIS3 ras1::TRP1 ras2::HPHr bcy1::KANr [CDC24-GFP] MATα</i>	This work
YRQ411	<i>RAS2/RAS2-Venus^C-TRP1 CDC24/CDC24-Venus^N-HIS3 MATα/α [pGAL-GST]</i>	This work
YRQ410	<i>RAS2/RAS2-Venus^C-TRP1 CDC24/CDC24-Venus^N-HIS3 MATα/α [pGAL-GST-RAS2]</i>	This work
YRQ400	<i>RAS2-Venus^C-TRP1 CDC24-Venus^N-HIS3 MATα</i>	This work
YRQ401	<i>alk1::NATr alk2::KANr RAS2-Venus^C-TRP1 CDC24-Venus^N-HIS3 MATα</i>	This work
YRQ397	<i>CDC24-Venus^N-HIS3 MATα</i>	This work
YRQ420	<i>RAS2-Venus^C-TRP1 MATα</i>	This work
yRQ73	<i>[GFP-RBD3] MATα</i>	This work
yRQ74	<i>alk1::NATr alk2::HIS3 [GFP-RBD3] MATα</i>	This work
yRQ262	<i>[GFP-RBD3][GAL-CDC42] MATα</i>	This work
yRQ263	<i>alk1::NATr alk2::HIS3 [GFP-RBD3][GAL-CDC42] MATα</i>	This work
yRQ264	<i>[GFP-RBD3][GAL-CDC42-G12V] MATα</i>	This work
yRQ265	<i>alk1::NATr alk2::HIS3 [GFP-RBD3][GAL-CDC42-G12V] MATα</i>	This work
yRQ84	<i>[CDC25-GFP] MATα</i>	This work
yRQ85	<i>alk1::NATr alk2::HIS3 [CDC25-GFP] MATα</i>	This work
yRQ93	<i>ira1::LEU2 ira2::URA3 MATα</i>	This work
yRQ95	<i>alk1::NATr alk2::HIS3 ira1::LEU2 ira2::URA3 MATα</i>	This work

yRQ29	<i>ras2Δ MATa</i>	This work
yRQ358	<i>ras1::TRP1 ras2::HPHr bcy1::KANr MATa</i>	This work

Plasmids used in this work

NAME	RELEVANT GENOTYPE	SOURCE
pRQ23	<i>pRS314-pACT1-GFP-BUD6-BNI4ter</i>	This work
pPD7	<i>pRS314-pACT1-GFP-BUD6-BNI4-BNI4ter</i>	This work
pRQ24	<i>pRS314-pGAL1-CDC42</i>	This work
pRQ25	<i>pRS314-pGAL1-CDC42-G12V</i>	This work
pRB2190	<i>pACT1-GFP-BUD6</i>	D.Botstein ⁷¹
pYS37	<i>pRS315-CDC24-GFP</i>	M.Peter ⁷²
pEG(KT)	<i>pGAL1-GST</i>	R.J.Deschenes
pRQ1	<i>pEG(KT)-RAS2</i>	This work
pYX242-		
GFP-RBD	<i>pYX242-eGFP-3RBD</i>	E.Martegani ⁶⁷
yEPCDC25e		
GFP	<i>CDC25-GFP</i>	E.Martegani ⁷³

Western blot

To analyze proteins during nocodazole treatment, cells were grown in YPD medium, synchronized in G1 with α -factor (2 μ g/ml), and released in the presence of nocodazole (10 μ g/ml). At given time points, samples were collected to obtain total protein extracts that were resolved by SDS-PAGE and analyzed by western blotting using proper antibodies (A-6455 for GFP, Ab6160 for tubulin), as previously described⁷⁴. Images were taken with a ChemidocTouch Imaging System (Bio-Rad) and

processed with ImageLab and ImageJ.

Protein localization assessment

Cells were synchronized as previously described, fixed with formaldehyde (3.7%) and washed 3 times in PBS⁵⁹. Localization was determined with a Leica DMRA2 widefield fluorescence microscope; images were processed with ImageJ. The centroid to centre of mass distance was calculated on sixty cells per strain using ImageJ and normalized on the daughter area, statistical significance was determined with a T-test (see fig.S8A). Signal intensity on the cell membrane was quantified as follows. Fluorescence intensity on the cortex of 60 daughter cells from 3 independent experiments was measured. Each cell was divided in 100 parts of the same length, and their intensity was normalized to the total fluorescence of the cell. The average intensity of each fraction was calculated as the mean of normalized fractions from all cells using the following equation, where I , i , j , n and m represent the intensity, the fraction, the cell, the number of analysed daughters and the number of fractions respectively (for further details see fig.S8B and C).

$$\bar{I}_i = \bar{I}_{m+1-i} = \frac{\sum_{j=1}^n \frac{I_{i,j} + I_{m+1-i,j}}{2 \sum_{i=1}^m I_{i,j}}}{n}$$

To determine the membrane/cytoplasm ratio of Cdc24 ROI were traced around 60 cell membranes per strain and the area and intensity of the ROIs were measured with ImageJ. The cytoplasm intensity was determined eroding the ROIs by 5 pixels and normalizing the raw

intensity on the area. To measure the intensity of the membrane, the same ratio was calculated by subtracting to intensity and area of the outer ROIs to that of their inner counterparts.

Actin Staining

Cells were grown as described, fixed with formaldehyde (3.7%), and washed three times with PBS. After incubation for 45 min with Alexa Fluor 594-conjugated phalloidin, actin was visualized by fluorescence microscopy.

Determination of Incorrect Anaphase

Cells were synchronized in G1 and released in nocodazole as described above. After 150 min. in nocodazole, cells were washed and released in fresh medium without the drug. At the indicated times after removal of nocodazole, cells were fixed with ethanol 100%, washed three times with PBS and stained with DAPI.

Bimolecular Fluorescence Complementation (BiFC)

Interaction between Cdc24 and Ras2 was determined by reconstitution of a functional YFP variant (Venus) by tagging genomic Cdc24 with Venus^N and Ras2 with Venus^C. Interaction was assessed by FACS analysis with a FACScan cytofluorimeter, measuring the fluorescence of a total 60000 cells from 3 independent experiments. The specificity of the approach was assessed by overexpression of GST-Ras2 under the control of a Gal promoter after 1.5 hours of Galactose addition. Evaluation by eye of the incidence and distribution of the arising signal using bud size as a marker for cell cycle progression was performed on

100 cells per categories per experiment.

Concanavalin A Staining

Cells grown in YPD were washed with PBS and resuspended in 125 μ l of AlexaFluor 488-conjugated concanavalin A (ThermoFisher C11252) at a concentration of 40 μ g/ml in the dark at room temperature. After 10 min, cells were washed and resuspended in appropriate medium for 1 hour, prior to nocodazole treatment.

Cell cycle analysis with FACScan

Samples were taken at given time points, fixed with ethanol and processed with RNase A and Proteinase K. Cells were then stained with 1 μ M SytoxGreen and DNA content was determined using a FACScan cytofluorimeter.

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Author contributions

R.Q., M.M.F. and P.P. planned the experimental approach, revised the experiments, analyzed the data and wrote the manuscript, R.Q. performed the experiments, M.G., E.G., G.R., G.R.G. and D.P. contributed to experimental procedures and discussion.

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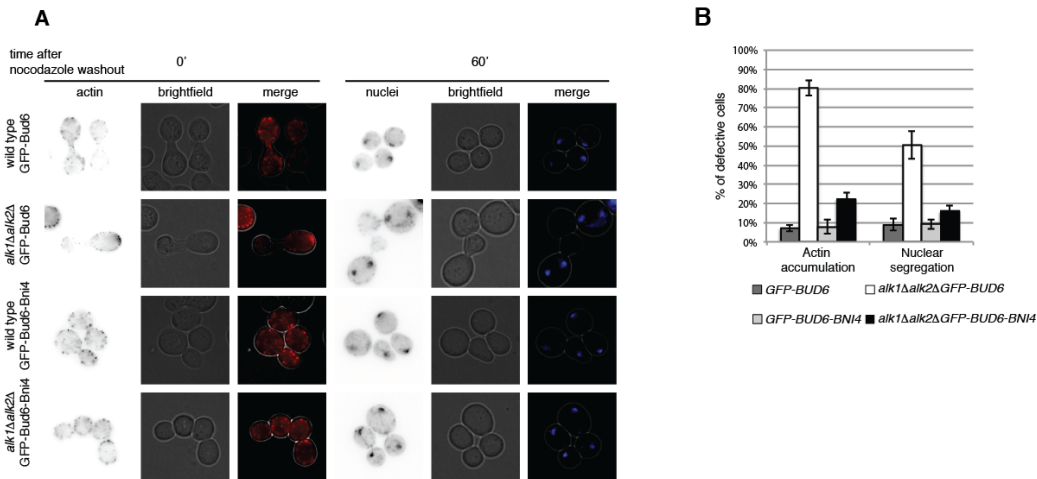


Figure 1 Loss of Bud6 causes actin and nuclear uneven distribution. **a,b** wild-type or haspin-lacking cells expressing either GFP-Bud6 or GFP-Bud6-Bni4 were presynchronized in G1 and then released in nocodazole-containing media. After 2.5 hours cells were released and samples were taken to monitor actin distribution (at 0' after the release) or nuclear segregation (60' after the release). Impact of Bud6-Bni4 chimera on actin distribution and nuclear segregation are shown in graph **b**.

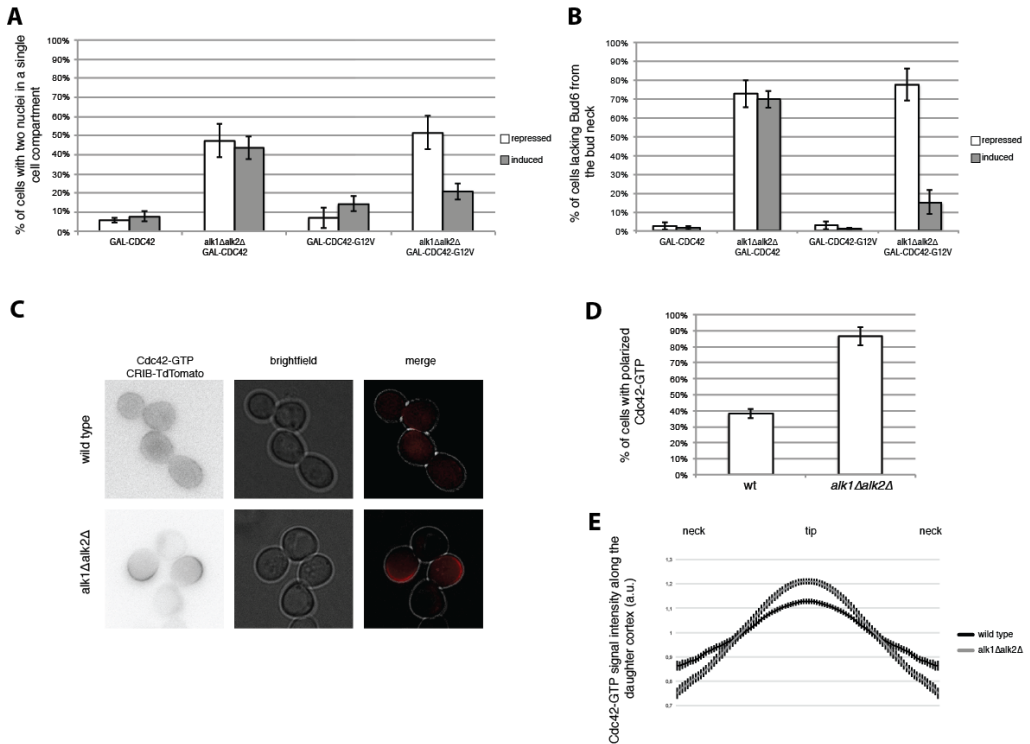


Figure 2 Haspin mediated regulation of Cdc42 activity is required for M-phase tolerance. Wild type or hyperactive Cdc42 was inserted in control or *alk1Δalk2Δ* cells under control of the galactose promoter and the effect of induction of the GTPase on nuclear segregation and Bud6 localization was assessed after an M-phase delay (a,b). Wild-type or haspin-lacking cells bearing CRIB-TdTomato (to assess localization of Cdc42-GTP) were treated with nocodazole after a G1 synchronization and analysed in fluorescence microscopy (c), scoring the percentage of cells with polarized Cdc42-GTP (d). The average CRIB-tomato signal intensity along the PM was quantified on 60 cells per strain (e); error bars represent standard error.

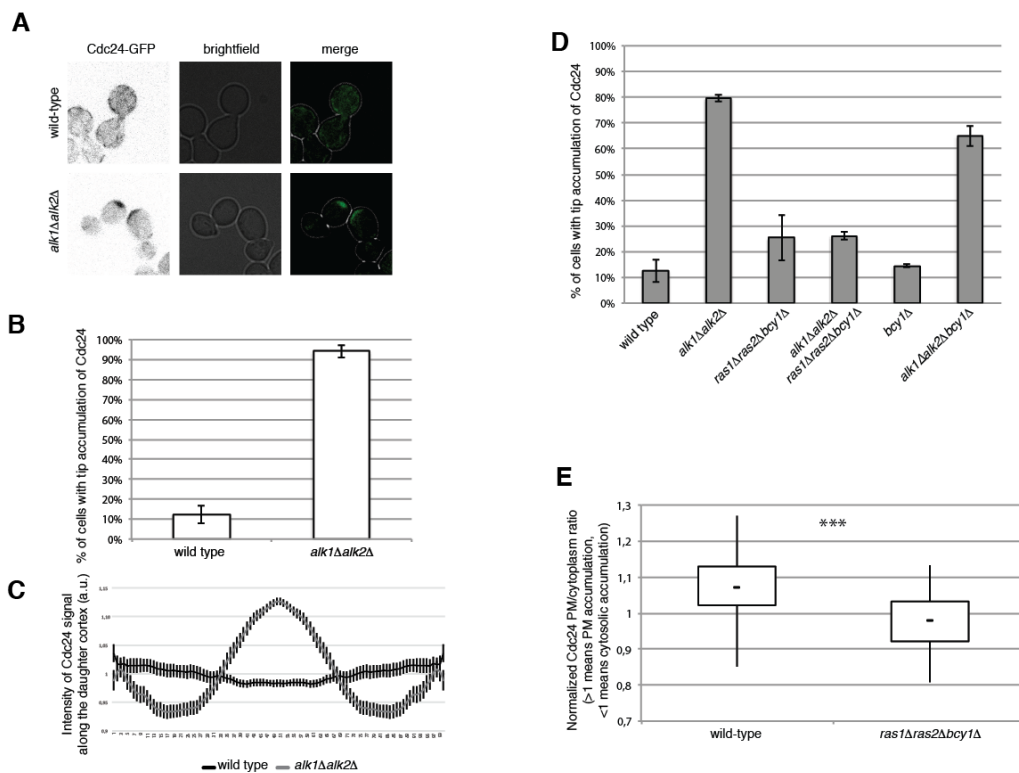


Figure 3 Localization of Cdc24 relies on haspin and Ras. **a,b,c** After presynchronization in G1, wild-type or haspin-lacking cells were arrested in nocodazole and the localization of Cdc24-GFP was evaluated, scoring cells with accumulation of the GEF at the bud tip **b** and quantifying the signal intensity along the PM **c**. Graph in **d** represents the percentage of cells with polarized Cdc24 in given strains after a 3 hours nocodazole treatment. Graph in **e** represents the ratio of normalized Cdc24-GFP fluorescence intensity between membranes and cytoplasm of given strains after 3 hours of nocodazole treatment (see material and methods for further details). Boxes include 50% of data points, line represents the average distance and whiskers report the minimum and maximum values.

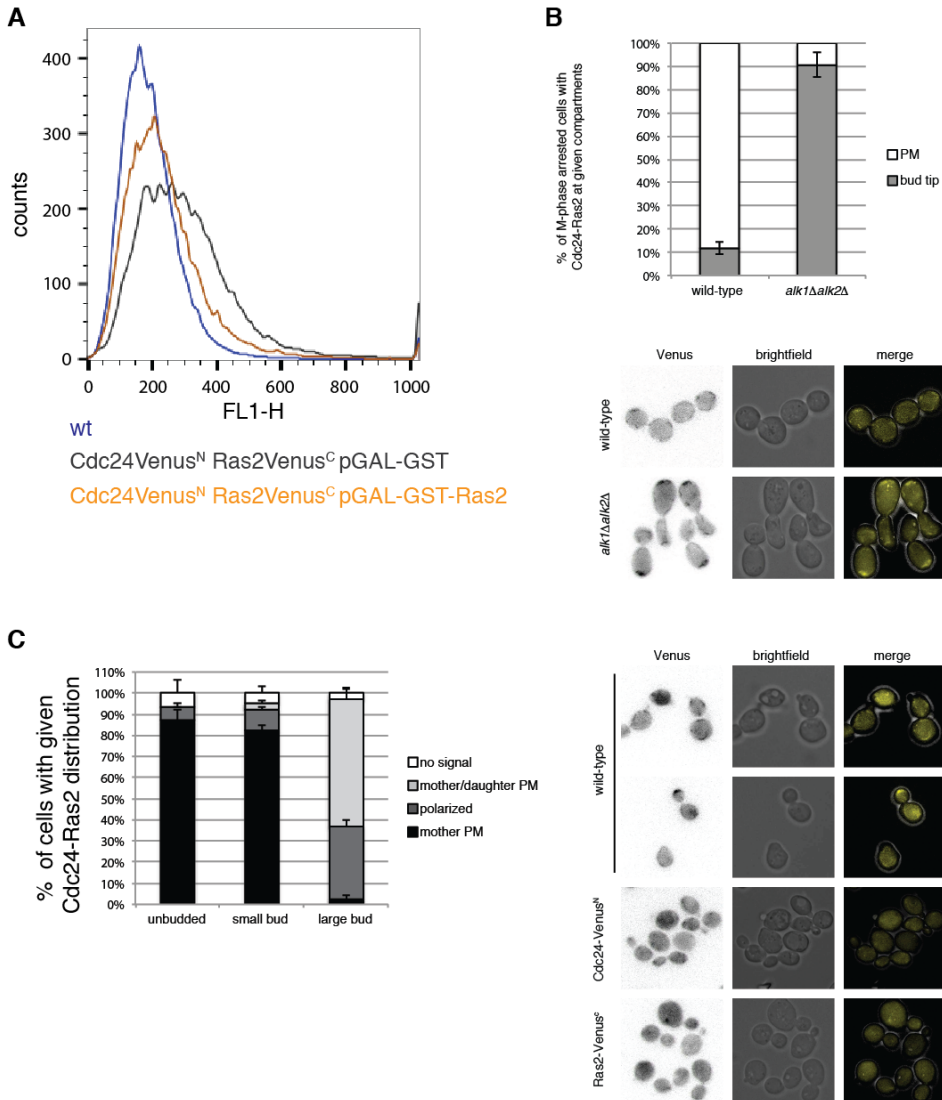


Figure 4 Cdc24 physically interacts with Ras2. Logarithmically growing cells were grown in raffinose and incubated for 1.5 hours with galactose. **a** Fluorescence intensity from 60000 cells from 3 independent experiments was measured in flow cytometry. **b** Cells were synchronized with mating factor and released in nocodazole. after 2.5 hours cells were fixed with formaldehyde and resuspended in 7.4pH buffered solution. Graph shows the percentage of cells with polarized Cdc24-Ras2; error bars represent standard deviation. **c** Graph represents the distribution of Ras2-Cdc24 in exponentially growing cells; for each experiment 100 cells per category were measured, error bars represent standard deviation.

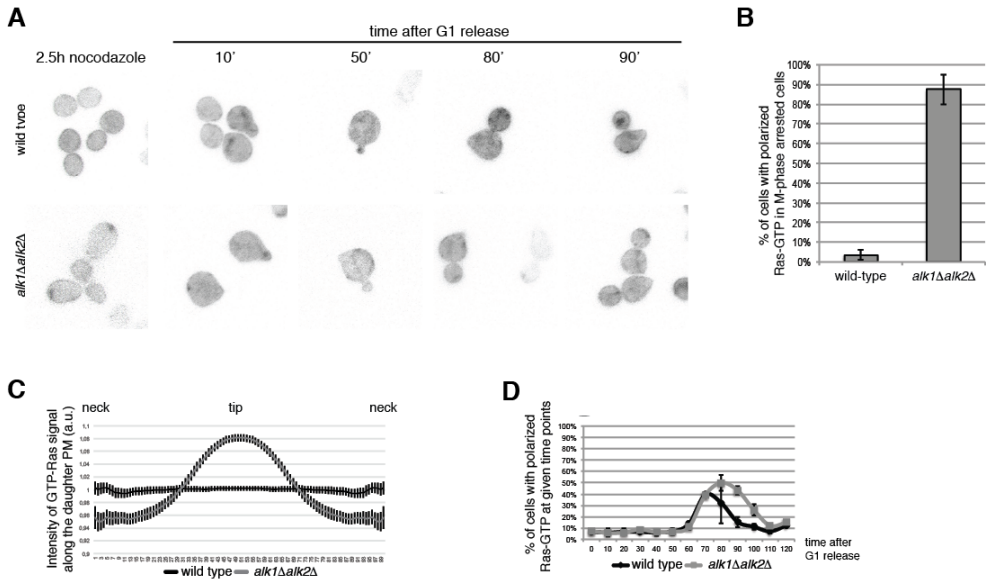


Figure 5 Haspin regulates active-Ras dynamics. The localization of Ras-GTP was evaluated exploiting the GFP-RBD probe in wild-type or haspin-lacking. Panel **a** shows examples of the phenotype at given time points. Graphs **b** and **d** show the percentage of cells with polarized active Ras during nocodazole treatment or in an unperturbed G1-G1 progression respectively; error bars represent standard deviation. The average intensity of GFP-RBD signal along the daughter cell cortex was then quantified from 60 cells for each strain and plotted in graph **c**, error bars represent standard error.

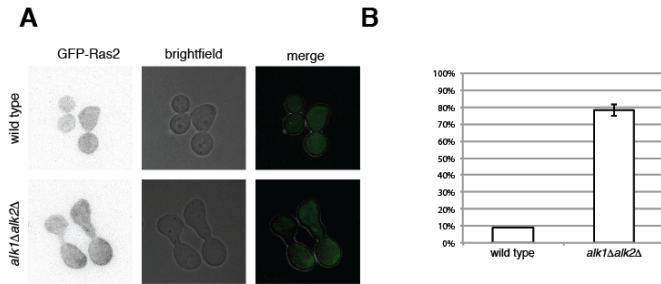


Figure 6 Localization of Ras is regulated by haspin. Cells were arrested in G1 and then released for 2.5 hours in nocodazole. After 1h45m in nocodazole, galactose was added to induce expression of GFP-Ras. At the end of the treatment cells were fixed and the percentage of cells with polarized Ras2 scored **a,b**.

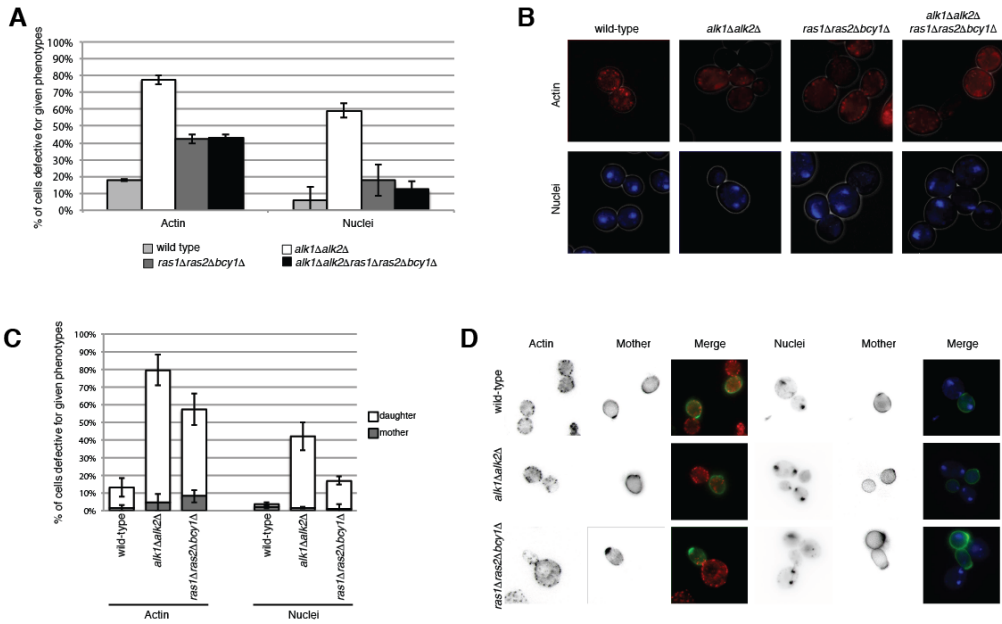


Figure 7 Ras is required for M-phase delay tolerance. **a,b** Cells of indicated strains were treated for 3 hours in nocodazole. At the end of the treatment, the drug was washed out and actin ($t=0'$ from the release) and nuclear segregation ($t=60'$ from the release) were monitored. **c,d** Cells were stained 10' with ConA-488, growth for an hour and then processed as in the previous experiment. Staining with ConA was exploited to distinguish mother and daughter cells.

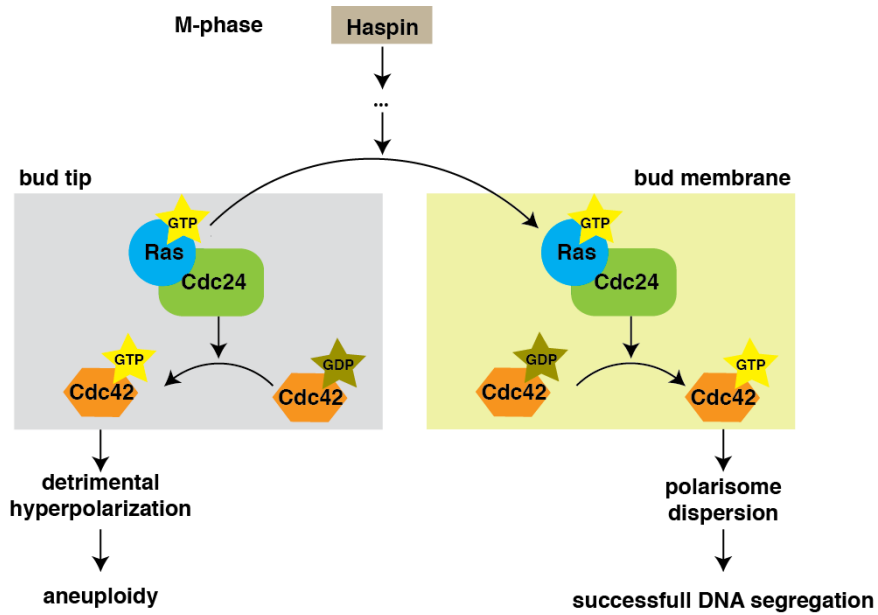
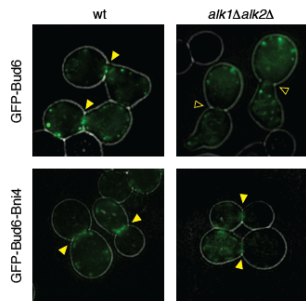
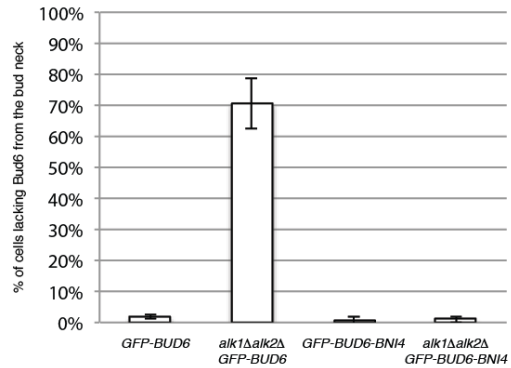
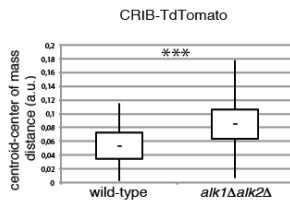


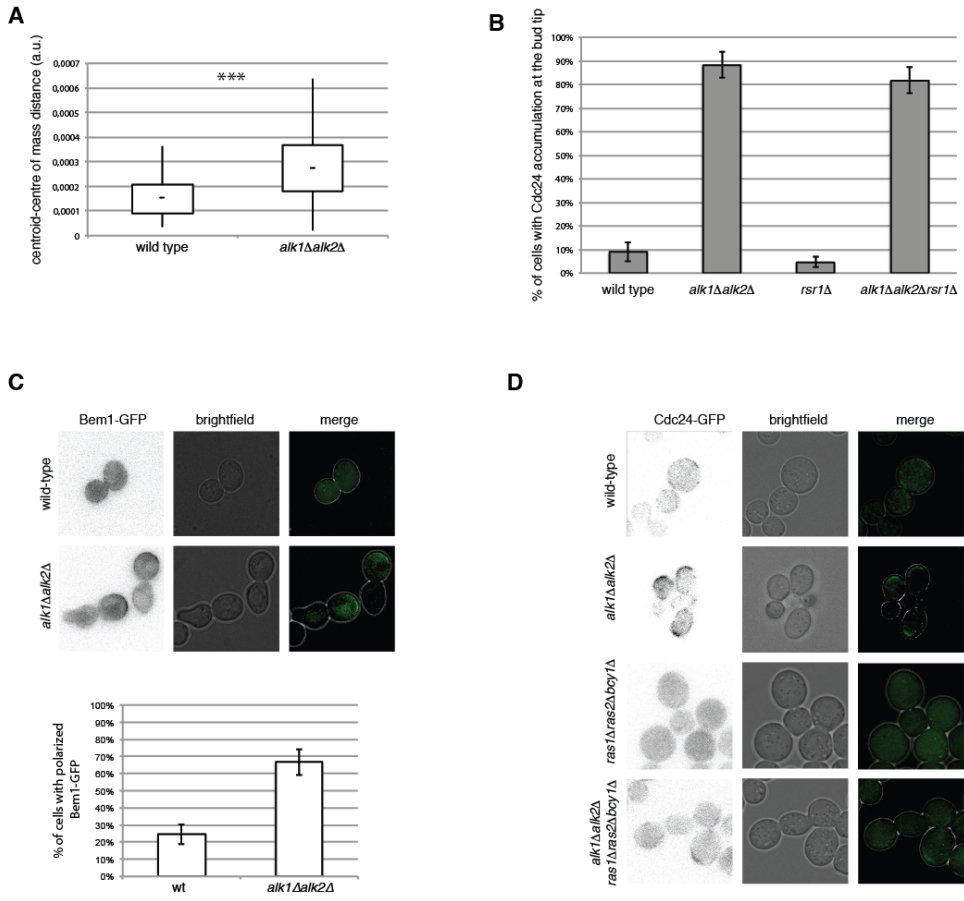
Figure 8 Ras dispersion from the bud tip is essential for a successful mitosis. During M-phase, haspin activity is required to redistribute active Ras from the bud tip, where it accumulates during bud growth, to promote an even distribution of the protein along the daughter cell membrane. Ras-GTP physically interacts with Cdc42 GEF, Cdc24, to promote a relocation of Cdc42 activity. If the cluster of active Ras is not dispersed from the bud tip, cells experience an excessive accumulation of polarity determinants at this region that eventually result in errors in chromosome segregation and aneuploidy.

A**B**

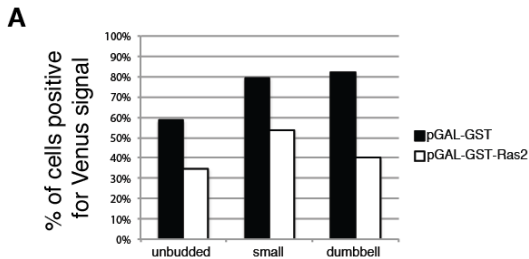
Supplementary Figure 1 a,b Localization of GFP-Bud6 and GFP-Bud6-Bni4 constructs in wild-type or haspin-lacking cells during a mitotic arrest. **a** Close arrows and oen arrows indicate presence or absence of the protein at the bud neck respectively. Graph **b** shows the percentage of cells lacking Bud6 from the bud neck, error bars represent standard deviation.

A

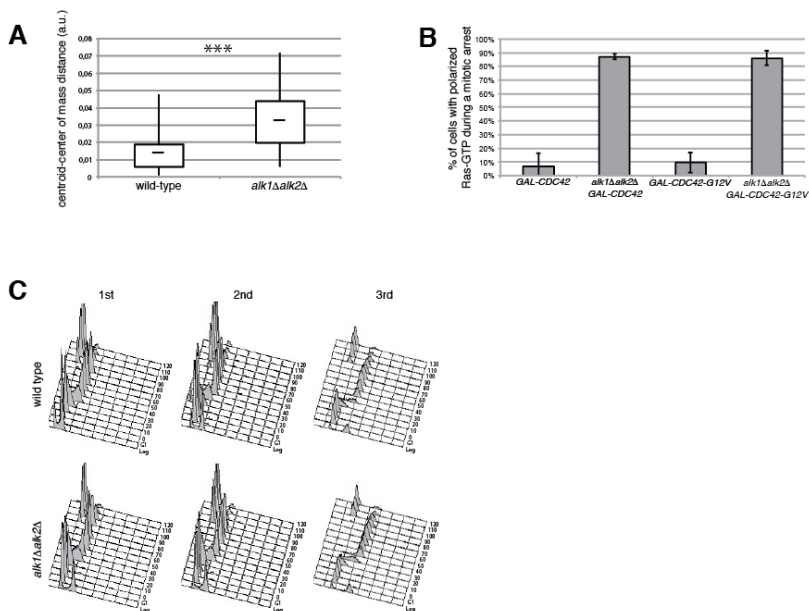
Supplementary Figure 2 a Plot represents the centroid-center of fluorescence mass distance normalized on the cell area, calculated on 60 cells per strain; boxes include 50% of data points, line represents the average distance and whiskers report the minimum and maximum values. *** pvalue<0,001.



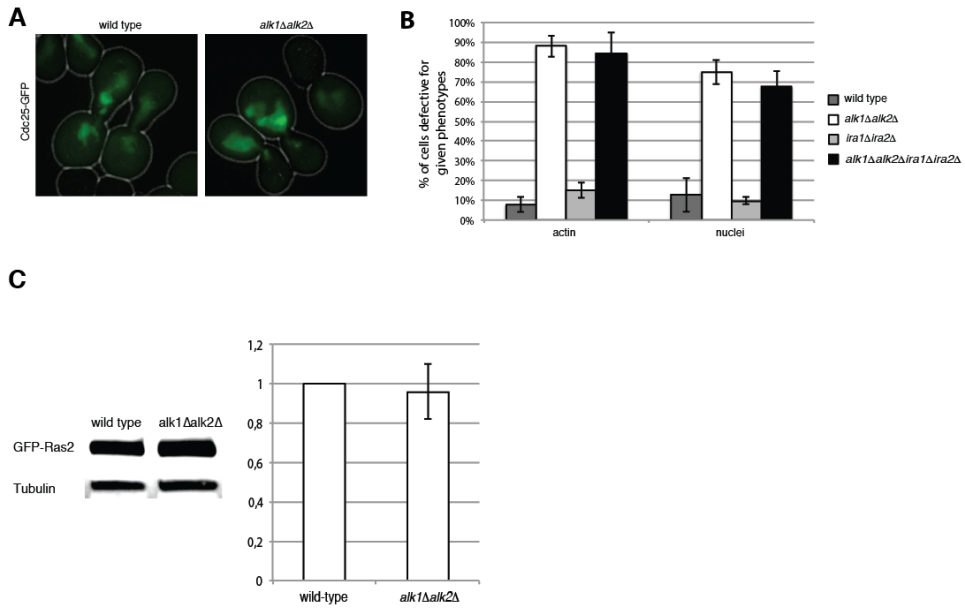
Supplementary Figure 3 Graph in **a** shows the centroid-centre of mass distance, normalized on daughter area of 60 cells treated as in Figure 2a. Boxes include 50% of data points, line represents the average distance and whiskers report the minimum and maximum values. *** p value $<0,001$. In **b** is reported the percentage of cells of given strains with polarized Cdc24 after presynchronization in G1 and 2.5 hours of nocodazole treatment. Assessment of Bem1-GFP in nocodazole-arrested wt or control cells is reported in **c**; error bars represent standard deviation. Sample images of experiment described in Figure 2d are shown in panel **d**.



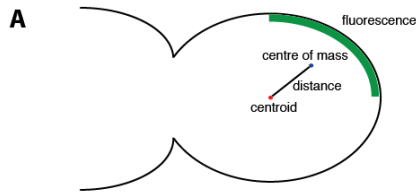
Supplementary Figure 4 Panel shows the percentage of logarithmically growing cells of given strains positive for Venus signal at given cell-cycle stages.



Supplementary Figure 5 Graph in **a** shows the centroid-centre of mass distance, normalized on daughter area of 60 cells treated as in Figure 5b. Boxes include 50% of data points, line represents the average distance and whiskers report the minimum and maximum values. **b** cells of given strains bearing the *GFP-3RBD* construct were growth on raffinose, arrested in G1 and released in nocodazole-containing medium. After 2 hours from the release galactose was added to induce Cdc42 overexpression and after another hour the localization of active Ras was evaluated. Panel **c** shows the cell-cycle progression of cells in a G1-G1 cell cycle, as described in Figure 4d.



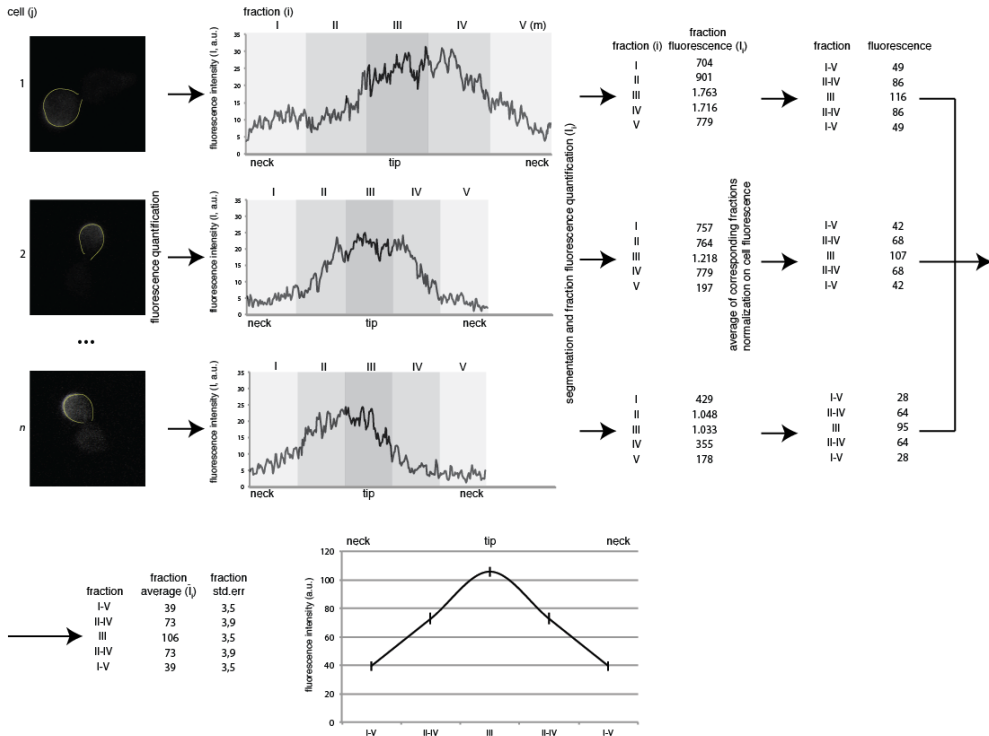
Supplementary Figure 6 a-c Cells of indicated strains were arrested in G1 and then released for 2.5 hours in nocodazole. Panel **a** shows the localization of Cdc25 in wild-type or haspin-lacking cells, graph **b** reports the impact of IRA1 and IRA2 deletion on actin and nuclear segregation. In panel **c** is shown the induction control of GFP-RAS2 for experiment in Figure 6.



B

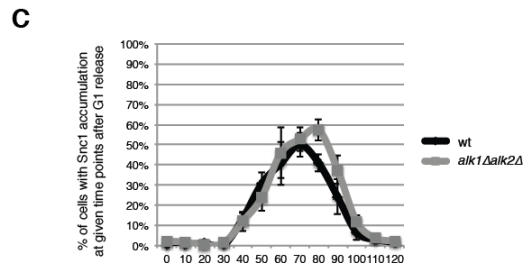
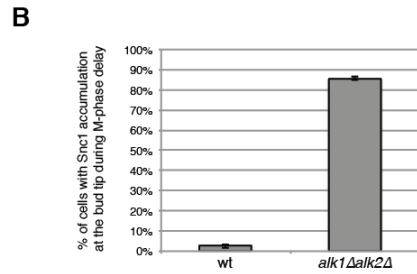
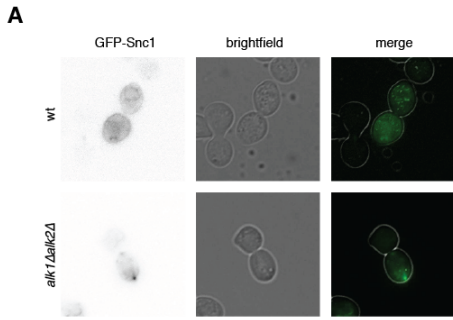
$$\bar{I}_i = \bar{I}_{m+1-i} = \frac{\sum_{j=1}^n \frac{I_{i,j} + I_{m+1-i,j}}{2 \sum_{i=1}^m I_{i,j}}}{n}$$

C



Supplementary Figure 7 Fluorescence quantifications. Figure **a** schematically represents the centroid-centre of mass distance. To measure the average fluorescence intensity, each cell cortex was divided in 100 parts of equal length and the intensity of each fraction was determined as the sum of all the pixels corresponding to that section. The average intensity of a given fraction was determined using the equation in panel **b**, in which I represents the intensity, i a fraction, j a cell, n the total number of cells (in our analysis 60) and m the total number of sections (in our analysis 100). Polarization was determined also as the distance between the geometric centre (centroid) of the cell and its fluorescence centre of mass. Panel **c** shows an application of equation B in which $n=3$ and $m=5$.

SUPPLEMENTARY INFORMATIONS



Loss of haspin causes polarized Snc1 accumulation. **a,b** wild-type or haspin-lacking cells bearing GFP-Snc1 construct were presynchronized in G1 and then released in nocodazole-containing media. After 2.5 hours samples were taken to monitor GFP-Snc1 distribution. Graph **b** shows the percentage of cells with polarized accumulation of Snc1. **c** Cells were arrested in G1 and release in an unperturbed cell-cycle, scoring the percentage of cells with polarized GFP-Snc1 signal every 10' after the release. Error bars in **b** and **c** represent standard deviation.

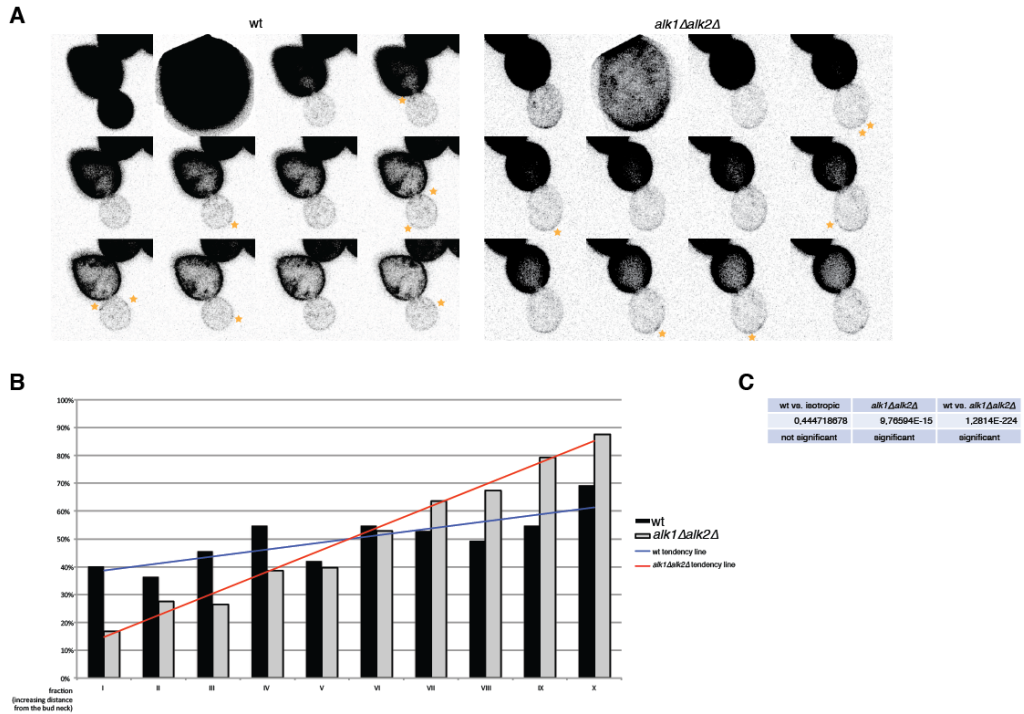
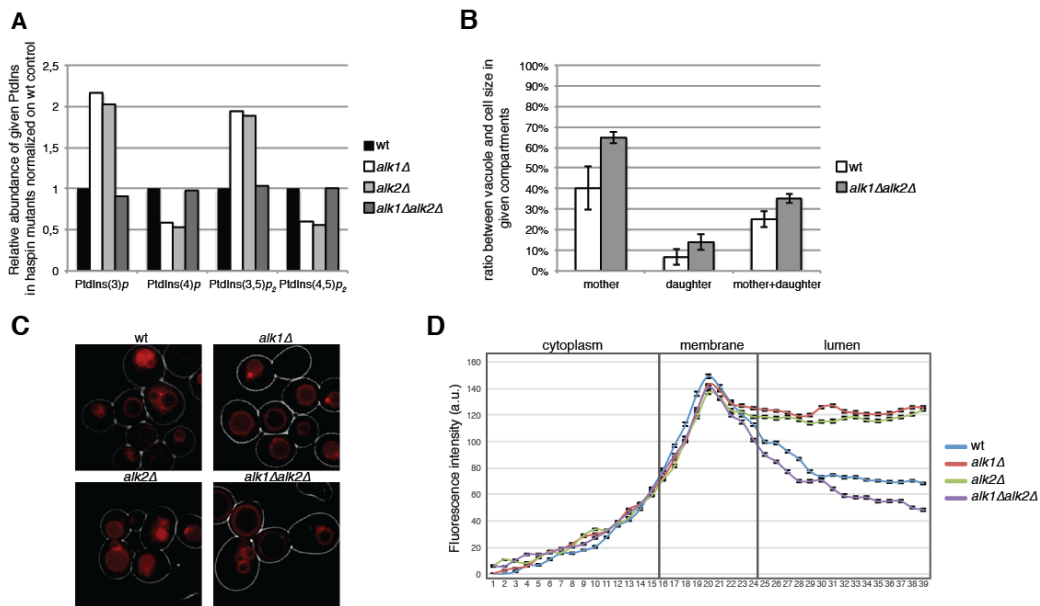
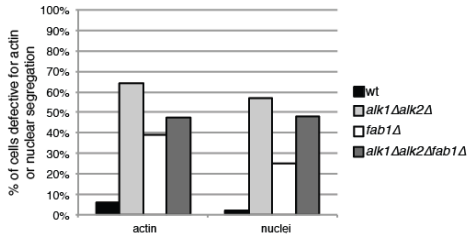
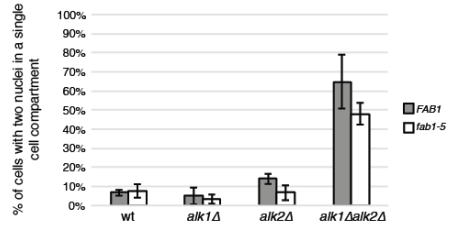
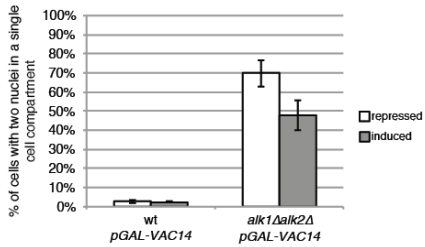


Figure 2 Vesicles are preferentially delivered towards the bud tip upon haspin loss **a,b** wild-type or haspin-lacking cells bearing GFP-Snc1 construct were presynchronized in G1 and then released in nocodazole-containing media. After 2.5 hours signal on the daughter cell was bleached and delivery of new vesicle was monitored in a time-lapse taking 1 picture every 3 seconds. Figure **a** shows delivery in wt or *alk1Δalk2Δ* cells, stars highlight position of fusion events. Graph in **b** reports the percentage of vesicles delivered to given fraction on the daughter PM (I is proximal to the bud neck, X is the bud tip), blue and red line indicate tendency line for wt or haspin-lacking cells respectively, the statistical significance of experimental distribution compared between the two strains and a theoretical isotropic distribution is given in **c**.



Haspin regulates PtdIns balance and vacuolar dynamics. Panel **a** shows the relative amount of different PtdIns in logarytmically growing wt or haspin mutants. **b,c,d** Wt or haspin-lacking cells were stained with FM4-64, presynchronized in G1, released in nocodazole and, at the end of the treatment, vacuolar morphology was assessed to determine the ratio between vacuolar and cellular area (**b**), or the fluorescence intensity profile across the vacuolar membrane (**d**).

A**B****C**

Haspin interplay with Fab1 complex. **a,b** Cells of given strains were arrested in G1 and released in nocodazole-containing medium. After 2.5 hours cells were shifted to nocodazole-free medium and actin (0' after the release) or nuclear segregation (60' after the release) were assessed. **c** Cells were grown in raffinose containing medium, arrested in G1 and released in nocodazole-containing medium for 3 hours. 1 hour before the end of the treatment galactose was added to induce overexpression of Vac14. At the end of the induction cells were released in fresh medium and nuclear segregation was assessed 60' after the release.