UNIVERSITÀ DEGLI STUDI DI MILANO

PhD COURSE IN MOLECULAR AND CELLUAR BIOLOGY

XXIX CICLO

Yeast haspin kinase regulates mitotic cell cycle events: from G2/M transition to polarisome dispersion

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PhD Thesis

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ACADEMIC YEAR: 2015-2016

“The important thing is not to stop questioning. Curiosity has its own reason for existing.”

Albert Einstein

SSD: [BIO/11]

Thesis performed at Dipartimento di Bioscienze

Contents

Part I

ABSTRACT

Haspin is a serine/threonine atypical kinase that phosphorylates histone H3-T3 during metaphase, promoting the recruitment of the chromosomal passenger complex (CPC) at kinetochores. Haspin depletion leads to cell arrest in mitosis and prevents proper chromosome positioning at the metaphase plate. *Saccharomyces cerevisiae* genome encodes for two haspin paralogues *ALK1* and *ALK2.* We recently showed that these genes are essential to coordinate polarization and cell cycle progression, ensuring the correct positioning of several polarity factors following a transient mitotic delay. The aim of this project is to identify new processes where haspin kinase is involved.

The first part of this work shows that Alk1 has a role at the G2/M transition in *S. cerevisiae.* These findings constitute the first evidence for Alk1-specific functions that are not shared by its paralogue Alk2. Our results indicate that cells lacking *ALK1* are sensitive to Latrunculin A and complete nuclear division within the unbudded mother cells. These observations pointed toward a defect in the morphogenesis checkpoint. We also observed that in absence of *ALK1* the Cdc28-Y19 phosphorylation signal decreases significantly during a morphogenetic stress. Exploring the underlying mechanism, we found that the decrease in phosphorylation is caused by a misregulation in Mih1 phosphatase activity in absence of Alk1. Therefore in budding yeast Alk1 modulates G2/M cell cycle switch by regulating Mih1

activity.

The second part of this work is focused on exploring the role of Alk1 and Alk2 in polarisome dispersion. We show that the previously reported role of haspin in polarization relies on its ability to modulate Ras localization. Our observations are indicative for a mitotic role of Ras, which, by regulating Cdc24 redistribution, influences Cdc42 activation at polarized sites. These observations may help to shed light on alterations in cell polarity, which often constitute the molecular mechanism for cancer insurgence.

SOMMARIO

Haspin è la serina/treonina chinasi che, fosforilando l'istone H3-T3 durante la mitosi, garantisce il reclutamento del chromosomal passenger complex (CPC) ai cinetocori. Poichè il complesso del CPC è fondamentale per il corretto allineamento dei cromosomi sulla piastra metafasica, in assenza di haspin le cellule di mammifero non possono portare a termine questo processo, arrestandosi in mitosi. Il lievito gemmante *Saccharomyces cerevisiae* è uno degli organismi modello più utilizzati per lo studio degli aspetti inerenti al ciclo cellulare. Il suo genoma codifica per due paraloghi di haspin, *ALK1* e *ALK2*.

Studi precedenti in laboratorio hanno dimostrato che in questo lievito haspin è necessaria per coordinare il processo di polarizzazione con la progressione del ciclo cellulare, assicurando la corretta localizzazione di numerosi fattori di polarità durante un blocco mitotico transiente. Lo scopo di questo progetto è di identificare nuovi processi in cui la chinasi haspin è coinvolta, esplorandone nuove funzioni.

Nella prima parte di questa tesi abbiamo dimostrato che Alk1 ha un ruolo nella transizione G2/M di *S. cerevisiae*. Queste evidenze indicano per la prima volta che esiste una funzione di Alk1 non condivisa col suo paralogo Alk2. I nostri risultati mostrano che cellule mancanti di *ALK1* sono sensibili al trattamento con la Latrunculina A e completano la divisione nucleare all'interno della cellula madre, senza portare a termine il processo di

gemmazione. Le nostre osservazioni suggeriscono che queste cellule sono difettive nel checkpoint morfogenetico. A riconferma di tale ipotesi abbiamo dimostrato che in assenza di *ALK1* il livello fosforilativo della Cdc28-Y19 è visibilmente ridotto durante uno stress morfologico. Cercando di identificare il meccanismo molecolare responsabile dei fenotipi osservati, abbiamo scoperto che la riduzione nella fosforilazione di Cdc28 è causata da una errata regolazione della fosfatasi Mih1 in assenza di Alk1. Alla luce di questi dati abbiamo concluso che Alk1 modula la transizione G2/M di *S. cerevisiae* tramite la regolazione della fosfatasi Mih1.

La seconda parte di questa tesi si concentra sul chiarimento del ruolo di Alk1 e Alk2 nella dispersione dei fattori di polarità cellulare. Infatti, abbiamo osservato che il ruolo di Alk1 e Alk2 nella polarizzazione cellulare che avevamo descritto in precedenza si basa sulla capacità delle due proteine di regolare la localizzazione di Ras. Le nostre osservazioni suggeriscono l'esistenza di un ruolo mitotico per Ras, la quale, regolando la redistribuzione di Cdc24, influenza l'attivazione di Cdc42 ai siti di polarità. Questi risultati riconfermano un ruolo per haspin nel processo di polarizzazione cellulare e forniscono nuovi spunti per la comprensione dei dettagli molecolari alla base di alterazioni della polarità cellulare, che caratterizzano spesso il motivo per cui una cellula sana può trasformarsi in cellula tumorale.

STATE OF THE ART

The cell cycle is a genetically controlled process, which leads to cell duplication and division. It promotes the reproduction of unicellular organisms as well as the growth and development of multicellular ones. Cell cycle goal is the correct division of genetic material and organelles between mother and daughter cell. To achieve this, all steps of the cell cycle must occur in a strictly precise timing and order. Two major processes characterize cell cycle progression: genome replication and chromosome segregation, defined also respectively as S and M phases. These two fundamental steps are preceded respectively by two gap phases G1 and G2, which allow cells to prepare for processes occurring in the following phase.

1. Saccharomyces cerevisiae cell cycle

Saccharomyces cerevisiae, also known as budding yeast, is one of the most studied eukaryotic model organisms. It is a unicellular fungus and carries a genome of nearly 6000 genes in 12.5 Mbp of DNA on 16 linear chromosomes. *S. cerevisiae* is characterized by a well-established genetics, which, together with its fast duplication time, makes yeast a robust model system to study eukaryotic molecular processes.

The life cycle of *S. cerevisiae* alternates between haploid and diploid states. Haploid cells can have two different sexes MATa and MATα, whereas diploids can only be found as MATa/α. Each

haploid cell responds to the mating pheromone of the opposite mating type. Two haploid cells of opposite mating type can mate and fuse, generating a *MATa/*α diploid cell, which in turn is not able to mate. However diploid cells can propagate through two different mechanisms: mitosis or meiosis. In particular, diploid cells dividing through meiosis produce a protective structure called *ascus* containing four haploid spores, which can be isolated and propagated as haploid clones.

The cell cycle of *S. cerevisiae* starts in G1 with a round unbudded cell (Figure I1). After G1 budding yeast cells face an important cell cycle regulatory point, the so-called “START”, which controls the progression from G1 to S-phase. The passage through START can occur only if environmental conditions are optimal for growth, since after that passage cells are commited to enter S-phase and complete cellular division. Therefore the cell cycle progresses through START only if conditions of nutrient aviability and appropriate size are satisfied. In particular, the regulation through a minimum cell size achievement ensures that the etherogeneous progeny composed by large mother and small daughter cells is maintained constant in size during subsequent cellular divisions. After START, cells enter S-phase in which occurs DNA replication and duplication of the spindle pole body (SPB), the microtubule organizing center of budding yeast.

Moreover, at G1/S transition the mother cell goes through budding, the process of bud emission. After S-phase cells enter G2, in which the two SPBs separate and the nucleus moves

toward the mother-bud interface, the bud-neck. Lastly, cells enter M-phase, during which the mitotic spindle elongates, and the sister chromatids are separated and segregated between the mother and the bud. At the end of the cell cycle the two dividing cells are split during a process called cytokinesis.

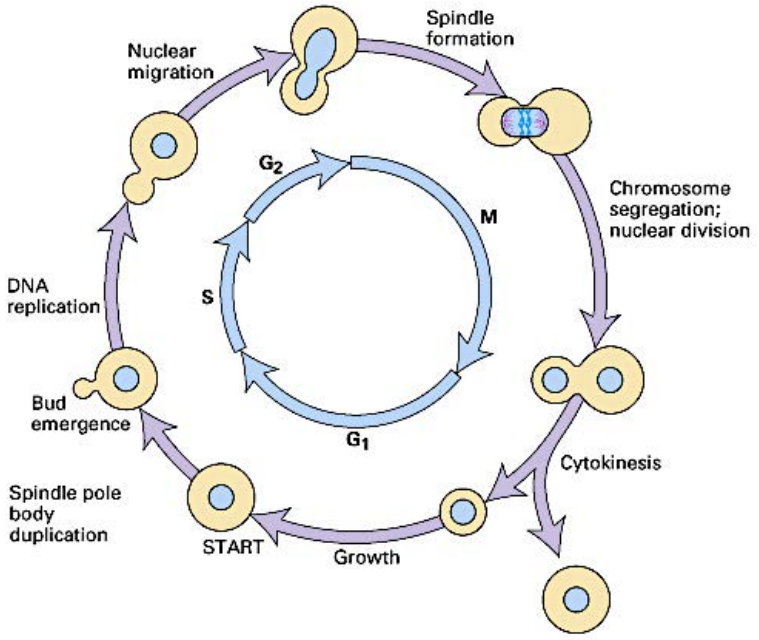

Figure I1. Cell cycle of the budding yeast *S. cerevisiae*[1].

The cell cycle is composed by four phases: G1, S, G2 and M. During G1 the cell grows until the fulfillment of G1 checkpoint, upon which can pass through START. After START a bud is emitted and S-phase begins. During S-phase the cell starts DNA and SPBs duplication. Upon G2 the nucleus migrates toward the bud and the spindle nucleates. Once chromosomes are aligned, the cell can enter M-phase, where is triggered chromosomes segregation.

2. Cell cycle regulation

Cell cycle progression is regulated by the controlled activation of cyclin-dependent kinases together with their cyclin binding partners. The fine modulation of these mechanisms is fundamental to avoid uncontrolled cell cycling, which can easily lead to carcinogenesis. Therefore the cell cycle is tightly regulated by CDKs (Cyclin Dependent Kinases), a highly conserved family of protein kinases, which control the transition from a cell cycle state to another. CDKs activity requires the interaction with their regulatory factors, called cyclins.

S. cerevisiae codes for six CDKs: Cdc28, Pho85, Kin28, Srb10, Bur1 and Ctk1. The only essential one is Cdc28, also known as CDK1, while the others are involved in secondary pathways and share redundant functions with Cdc28[1-3]. *CDC28* encodes for a 34kDa serine-threonine kinase, which is finely regulated by the interaction with nine different cyclins (Figure I2).

These cyclins are of two different types: CLNs (Cln1, Cln2, Cln3), and CLBs (Clb1, Clb2, Clb3, Clb4, Clb5 and Clb6). CLN cyclins act in G1 and are necessary for the beginning of the cell cycle. CLB cyclins act in two principal moment of the cell cycle: Clb5 and Clb6 are involved in DNA replication during S-phase, whether Clb1-4 become fundamental for the assembly and function of the mitotic apparatus in M-phase[4-6].

To achieve the proper regulation of cell cycle usually CDKs are present in excess and stable in concentration along the cell cycle.

Given that, the regulation of CDKs activity is governed by precisely modulating the levels and availability of cyclins. Cyclins interaction with CDKs gives specificity for proper substrates, ensuring the commitment of different cell cycle events. For these reasons, cyclins expression and degradation is finely regulated during the cell cycle, ensuring that the series of event caused by their interaction with CDKs verify only with a proper timing and succession.

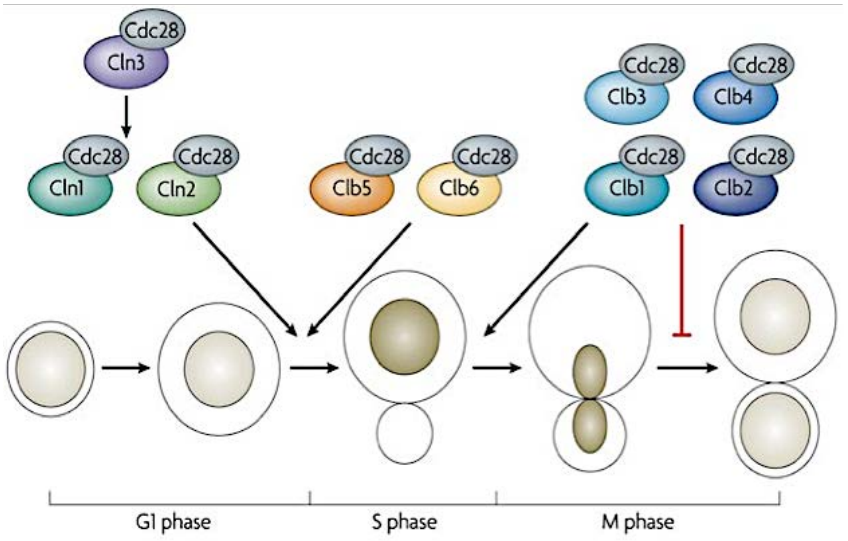

Figure I2. Cdc28-cyclins complexes in the budding yeast cell[7].
Cdc28 is activated by multiple cyclins. The G1-phase cyclins (Cln1, Cln2 and Cln3) promote budding, SPBs duplication and activation of the B-type cyclins. The S-phase cyclins (Clb5, Clb6) induce DNA replication and the M-phase cyclins (Clb1, Clb2, Clb3 and Clb4) promote spindle formation and mitosis onset.

3. Cell cycle checkpoints

Cell cycle events must be coordinated so that they occur in the proper order with respect to each other. Nature has developed surveillance pathways, which are known as cell cycle checkpoints, to ensure that cells progress through the cell cycle properly[8,9]. Checkpoints have a fundamental role in the maintenance of genomic and cellular integrity, since by their activation cells arrest cell cycle until key events for cell cycle progression have not been finely completed[10,11]. Every checkpoint regulates the fulfilment of a proper event, giving cells the chance to cope with stress conditions and several types of insults before proceeding in cycling. For example DNA replication checkpoint delays entry into mitosis until DNA has not been satisfactorily replicated, guarding against genome instability. Another essential checkpoint for cells is the spindle assembly checkpoint (SAC), which senses errors in kinetochores attachments to mitotic chromosomes, delaying entry into mitosis to avoid erroneous chromosome segregation.

A peculiar situation is the one of DNA damage checkpoint that differs from other checkpoints since it preserves genome integrity from DNA damages, which can occur at any time during the cell cycle. DNA damage checkpoint activity for instance, does not regulate the transition from a cell cycle phase to the subsequent; instead it delays cell cycle to give cells time to repair damages before they become unfixable. For these reasons this checkpoint can influence cell cycle progression in any moment of the cell

cycle, inducing a delay in G1, a slowdown of S-phase, an arrest in G2 or even a stall in mid-anaphase[12-15].

Another fundamental checkpoint for cells is activated upon damages detected at the G2-phase and is defined as the G2/M transition checkpoint. This checkpoint is fundamental since events of chromosome segregation depend upon CDK1 activity and mitotic cyclins concentration, which rises gradually from G2/M. If damages are present in this phase, CDK1 activation is inhibited by Wee1 kinase through a phosphorylation on Y15, impeding CDK1-cyclins interaction and delaying entry into mitosis[16-18]. Once all the conditions for G2/M transition are achieved, Cdc25 phosphatase removes Wee1-dependent CDK1 inhibitory phosphorylation triggering CDK-cyclins activation and mitotic entry[19-23]. Both *WEE1* and *CDC25* must be tightly regulated during cell cycle to allow a correct temporal and spatial regulation of CDK-cyclins complex activity. Overexpression of *WEE1* has indeed been observed in many tumors like glioblastoma, malignant melanoma, hepatocellular carcinoma or luminal and *HER-2* positive breast cancers. *CDC25* as well has been reported as overexpressed in various human cancers including lung, colorectal, prostate, ovarian, breast, hepatocellular, neuroblatoma, glioma, pancreatic and many more. Therefore the understanding of mechanisms that regulate *WEE1* and *CDC25* is of fundamental importance in cancer research.

4. The morphogenesis checkpoint of budding yeast

The pathways ensuring a correct transition between different phases of the cell cycle are fundamental for cells and have been conserved by evolution. In *S. cerevisiae* for instance, the G2/M transition is strictly regulated by the morphogenesis checkpoint (Figure I3). In particular, in *S. cerevisiae* this checkpoint becomes essential for cells viability only upon perturbation of actin polarity and cytoskeletal structures that impair bud formation. If sufficient growth of the bud has not succeeded cell cycle progression is arrested through an inhibitory phosphorylation at Cdc28-Y19, the budding yeast equivalent residue for the human Y15 of CDK1[24-27]. This event avoids that nuclear division proceeds in absence of a recipient daughter cell, preventing the formation of binucleated cells within a single cell compartment[28,29].

The inhibitory phosphorylation of Cdc28 is performed by the protein kinase Swe1, orthologue of human *WEE1*. Swe1 phosphorylation inhibits the activity of CLBs-Cdc28 complexes, preventing nuclear division[30]. Strains deleted for *SWE1* gene are unable to arrest before mitosis in response to actin perturbations and thus divide their nucleus within the mother cell, generating a binucleated population[31,32].

Morphogenesis checkpoint monitors actin organization, resulting in a cell cycle delay only if actin perturbation occurs during the critical early phase of bud formation[33]. Budding is a cellular

process that begins in G1 by the activation of CLNs–Cdc28 complexes[34].

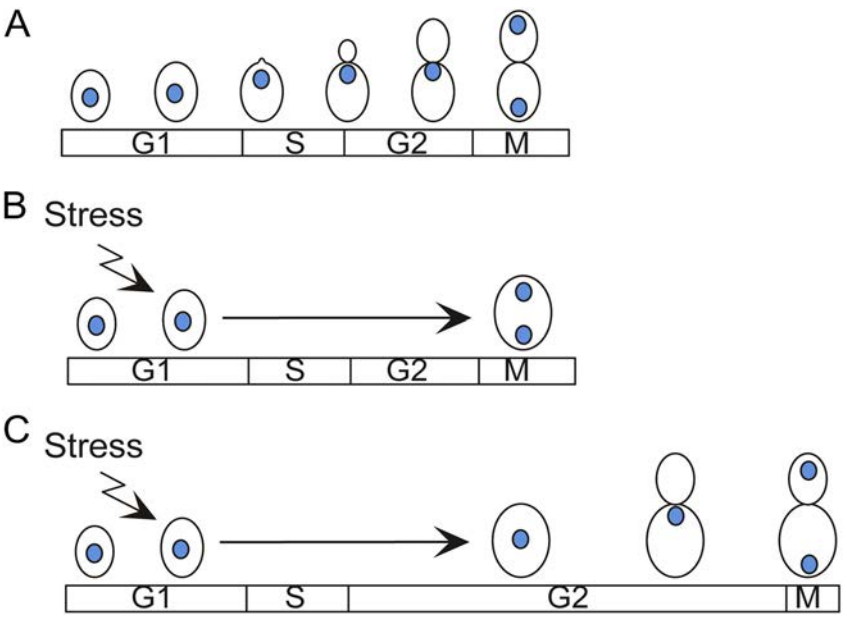

Figure I3. The morphogenesis checkpoint in *S. cerevisiae* [35]. **(A)** During an unperturbed cell cycle, bud formation and DNA replication happen simoultaneusly, ensuring that a bud is present to receive the daughter nucleus during nuclear division. **(B)** If the cell cycle goes on when budding is impaired, cells would become binucleated. **(C)** To avoid the situation shown in **(B)** the morphogenesis checkpoint ensures compensatory G2 delays of the cell cycle if bud formation is incomplete.

Moreover budding requires the activity of Cdc42, a Rho-family GTPase that plays a pivotal role in *S. cerevisiae* polarization (discussed below). Cdc42 activation is performed by Cdc24, the nucleotide exchange factor responsible for its GTP loading[36]. Temperature-sensitive mutants for cdc24 and cdc42 prevent

activation of Cdc42 at their restrictive temperature, resulting in the failure of cytoskeletal elements polarization and budding[34,37,38]. Same effects have been observed upon treatment with Latrunculin A (LatA), an actin depolymerizing drug that, by impeding actin assembly, induces the morphogenesis checkpoint activation[39-41].

Many works have focused on the ability of the morphogenesis checkpoint in inhibiting mitotic entry; however, the checkpoint is also responsible for delays during mitosis[42-46]. In particular, CDK1 is the final target of the checkpoint and its activity is required for anaphase onset, which depends on phosphorylation and activation of the anaphase promoting complex (APC/C)[47-51].

Recently it was shown that inducing a prolonged inhibitory phosphorylation on CDK1 causes a longer metaphase arrest, suggesting that the morphogenesis checkpoint proper arrest point is in metaphase. During this arrest the anaphase onset is inhibited since APC/C activation is the target of the checkpoint[46,52].

5. Swe1

In eukaryotic cells, entry into mitosis is induced by the formation and activation of CLB-cyclins and CDK1 complexes. As previously discussed, in higher eukaryotes CDK1 is negatively regulated by the Wee1-dependent phosphorylation at Y15, which is counterbalanced in cells by the activity of Cdc25 phosphatase[21,53]. Wee1 kinase is conserved also in budding yeast, where it is

encoded by *SWE1* gene[54,55]. Also in *S. cerevisiae* Swe1 kinase modulates Cdc28 through an inhibitory phosphorylation, which in this organism occurs on Y19. The activity of Swe1 is induced by morphogenetic stresses in a pathway known as the "morphogenesis checkpoint". This checkpoint ensures that nuclear division is triggered only if bud formation has occurred[34]. The precise aspect of morphogenesis that constitute the signal for checkpoint activation is still controversial and may consist in a combination of different factors such as bud emergence, bud growth, actin and septin organization[33,43,56-60]. Any disruption or alteration of actin cytoskeleton causes activation of this checkpoint, leading to a cell cycle arrest at the G2-phase with replicated DNA, thus preventing nuclear division. Once proper budding is completed, Swe1 must be degraded. Simultaneously the phosphatase activity of Mih1, the yeast ortholog of Cdc25, has to revert Cdc28-Y19 phosphorylation to allow entry into mitosis[18-22].

Swe1 regulatory pathway is fundamental to avoid that nuclear division occurs in presence of actin organization defects (Figure I4). In particular, Swe1 protein begins to accumulate from S-phase and starts to be subsequently phosphorylated with cell cycle progression, resulting in an hyper-phosphorylated form[44,61,62]. This form is the target of ubiquitination events, which trigger its degradation through the 26S proteasome[63,64]. Therefore Swe1 phosphorylation events are fundamental passages that couple Swe1 degradation with G2/M transition.

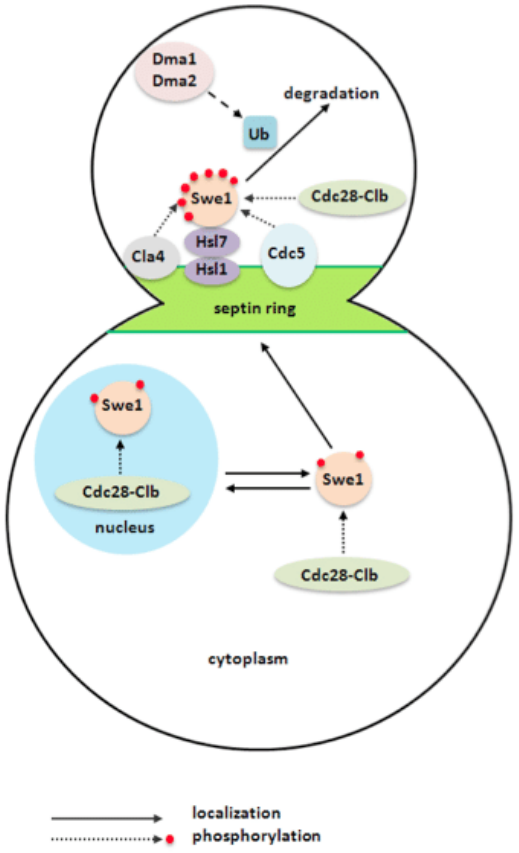

Figure I4. Swe1 regulation in *S. cerevisiae*[73].

Swe1 moves from the nucleus to the cytoplasm and is subsiquently recruited to the bud-neck. Changes in Swe1 localization are regulated by Cdc28-Clb kinase phosphorylations. Once at the bud-neck, Swe1 is targeted to Dma1 and Dma2-dependent ubiquitynation. Lastly the ubiquitinated form of Swe1 is degradated by the proteasome.

Regulation of Swe1 levels not only depends upon multiple kinases activity, but also requires the correct septin collar assembly. Indeed, septin filaments constitute the scaffold for Swe1 regulators recruitment at the bud-neck. In particular upon septin structure assembly Hsl1 and its scaffold Hsl7 are recruited at the

bud-neck, where they induce Swe1 recruitment through priming modifications[62,65-70]. Once at the bud-neck, Swe1 is directly phosphorylated by a PAK homologue Cla4 and the polo kinase homologue Cdc5, which both share the same bud-neck localization timing of Swe1. Specifically, it has been shown that Cla4 is responsible for early phosphorylation of Swe1, whereas Cdc5 acts to transform the low phosphorylated pull of Swe1 into a hyper-phosphorylated one[71,72].

Interestingly, it has been demonstrated that Cdc28-Clb2 complexes phosphorylate Swe1 *in vitro*, generating a recognition motif for further Cdc5-dependent phosphorylation[65]. This observation claims that a small amount of Cdc28 escapes inhibitory phosphorylation by Swe1, starting a feedback loop that triggers its reactivation through Cdc5-dependent Swe1 hyper-phosphorylation and subsequent degradation. This feedback mechanism is conserved by evolution in higher eukaryotes, where CDK1 and Plk1 act on common substrates for the regulation of many mitotic events.

As priory discussed, Swe1 protein is strictly regulated during cell cycle progression. Swe1 downregulation depends upon its phosphorylative status and constitutes the limiting step for mitotic entry. Only the hyper-phosphorylated form of Swe1 is recognized as substrate for ubiquitination and is subsequentially degraded by the proteasome[63]. In particular, it was found that the Met30/SCF complex ubiquitinates Swe1 *in vivo*, targeting the kinase to the Cdc34-dependent proteolysis network[64]. The subsequent

reduction in nuclear population of Swe1 allows Cdc28 activation coupled with mitotic progression. This mechanism is conserved also in mammalian cells, where Cdc2- and Plk1-dependent phosphorylation on Wee1 triggers protein degradation through the SCF complex[74]. Moreover, it was shown that loss of Cdc55 function causes Swe1 stabilization, claiming that also the protein phosphatase 2A (PP2A), in combination with its regulatory subunit Cdc55, is required for Swe1 degradation[75]. The network of pathways regulating Swe1 is extremly complex and the degradation of bud-neck pool of Swe1 still needs to be investigated in detail. Among the players of Swe1 regulations other two ubiquitin ligases have been found as responsible for Swe1 ubiquitination in budding yeast: Dma1 and Dma2. In particular, Dma proteins belong to the same FHA-RING ubiquitin ligase family of human Chfr and Rnf8 and they have a role in mitotic checkpoints, during the control of septin ring dynamics and in cytokinesis[76,77].

6. Mih1

Cdc25 phosphatase is responsible in fission yeast and many higher eukaryotes for re-activation of CDK1 at the G2/M transisiton[16-22, 78]. Since Cdc25 activity is fundamental for G2/M transition checkpoint, the protein results temporally and spatially regulated by various factors including CDK itself, ERK-MAP kinase, Plk1-Polo kinase, PP2A phosphatase, 14-3-3 and SCF ubiquitin ligase. Also fission yeast Cdc25 undergoes hyper-phosphorylation during mitosis[79,80]. Interestingly, CDK1

associated with mitotic cyclins directly phosphorylates Cdc25C *in vitro*, inducing a fourfold increase in phosphatase activity and suggesting the existence of a positive feedback loop for CDK1 activation *in vivo*[81,82].

Mechanisms of Cdc25 regulation are conserved by evolution in budding yeast, where Mih1 homologous phosphatase undergoes dramatic changes in phosphorylation throughout most of the cell cycle in a casein kinase 1-dependent manner[83]. These modifications depend also upon Cdc28 activity and keep Mih1 inactive along cell cycle progression. Only during G2/M transition Mih1 is dephosphorylated by Cdc55-dependent PP2A phosphatase with the help of Zds proteins, becoming able to remove the phosphate group on Cdc28-Y19 and other proper mitotic substrates[83,84]. Moreover, Mih1 presents a nuclear localization signal (NLS) constituted by a cluster of three lysines in its N-terminal region, which triggers its accumulation in the nucleus during telophase[33].

Interestingly, *MIH1* gene becomes essential if *HSL1* is deleted, since Swe1 is not recruited at the bud-neck and becomes stabilized. In these conditions the inhibitory phosphorylation of Cdc28-Y19 becomes constitutive, and Mih1 activity results fundamental for Cdc28 dephosphorylation and mitotic entry. These observations indicate that the G2-arrest derives from the concomitant stabilization of Swe1 protein and removal of Mih1 activity[86]. Moreover, evidences in budding yeast show that deletion of *MIH1* induces only mild delays in mitotic entry and

anaphase onset[51,53,83,86]. These observations were reconfirmed by the discovery that additional redundant phosphatase act on Cdc28-Y19 dephosphorylation. In particular, it was shown that Mih1, Ptp1, and $PP2A^{Rts1}$ act redundantly in a mechanism for the stepwise activation of CDK1 prior to anaphase onset[87].

Wee1 and Cdc25 have crucial activities in cell cycle control. This makes them very good candidates for the development of peculiar strategies to indirectly inhibit CDKs in cancer cells. Often cancer cells are mutated in *p53* and rely on the G2-checkpoint for DNA damage repair before entry into mitosis. The inhibition of Wee1 is already used in cancer therapy in combination with DNA-damaging agents to reduce cancer cells growth in various tumors[88]. Oppositely, the upregulation of Cdc25 activity could inhibit the DNA damage checkpoint pathway, pushing cells to enter mitosis before DNA repair. Currently it has not yet been identified a compound acting to increase the catalytic activity of Cdc25 phosphatase, leaving open questions in the *scenario* of anti-cancer therapeutics. The study of *WEE1* and *CDC25* as targets for cancer therapies is a huge field of investigation, but there is still a remarkable gap of molecular knowledge on these pathways that needs to be filled.

7. Saccharomyces cerevisiae mitosis

Mitosis is the process that leads to equal partitioning of replicated chromosomes into two dividing cells. As previously discussed, entry into this phase of the cell cycle is triggered in eukaryotic

organisms by the activation of the conserved CDK1-CLB cyclins complex homologues. In particular, in higher eukaryotes CDK1-CLB cyclins activation is regulated by the two opposing activities of Wee1 inhibitory kinase and Cdc25 activatory phosphatase. The same mechanism is also conserved in budding yeast and acts through Swe1 and Mih1 orthologues.

The maintenance of genomic identity between mother and daughter cell is guaranteed by the DNA replication during S-phase, which produces two identical sister chromatids. Sister chromatids are hold together by the cohesion complex (Smc1, Smc3, Scc1, Scc3) at the centromere and are divided during the process of mitosis. In higher eukaryotic cells, mitosis is composed by different phases (prophase, prometaphase, metaphase, anaphase and telophase) that follow one another to ensure a proper cellular division. Mitois starts with prophase, during which chromosomes condensate. The mitotic process goes on with prometaphase where the nuclear envelope breaks down in small vesicles and microtubule-chromatid interactions are established. Subsiquently, during the step of metaphase the tension generated by the cohesion between sister chromatids stabilizes their bipolar attachment to microtubules emanating from opposite poles of the dividing cell. Moreover, once all the chromosomes have properly attached to microtubules, the tension generated triggers chromosomes alignment equidistantly from the two opposite poles. Metaphase is followed by anaphase, during which an enzyme called separase splits the cohesin complex that holds tightly together sister chromatids, promoting chromosomes

segregation. Mitosis ends with telophase, where cell cycle events of prophase and pro-metaphase are reversed, and nuclear envelops are reconstituted around the two new nuclei. Lastly, the process of cytokinesis physically cleaves cellular membrane producing two identically daughter cells.

Since in higher eukaryotes nuclear envelope is reconstituted upon chromosomes separation, the process is defined as “open” mitosis. Fungi such as *S. cerevisiae* instead undergo a “close” mitosis, without nuclear envelope breakdown. This can happen since budding yeast peculiar SPBs are embedded in the nuclear membrane. In particular, also in this organism, at the beginning of metaphase, mitotic spindle microtubules are bound to the kinetochores and establish a connection between each sister chromatid and one of the two cellular poles. Each chromatid is thus connected to one of the two SPBs through microtubules interacting with its kinetochore. These microtubules apply a pulling force on the chromatids towards the spindle poles, while the cohesion between the sister chromatids opposes to this force. These balanced forces, acting on chromosomes, pull them on the *metaphase plate*, an imaginary line that is equidistant from the two SPBs. If the cell can not perceive the balance between these forces, the cell cycle arrests, preventing a premature progression to anaphase, until all chromosomes are aligned on the metaphase plate. Sister chromatids cohesion is essential for accurate chromosome segregation and is detected through several structural proteins[89-93]. The molecular basis for sister chromatid cohesion is the protein complex called cohesin. When the Scc1

subunit of the cohesin complex is cleaved by the separase Esp1, chromosome segregation is triggered[94].

The separase Esp1 is normally inhibited by the activity of the securin Pds1, which protects Scc1 and cohesion from cleavage. During anaphase the degradation of Pds1 allows Esp1 to cleave Scc1, promoting sister chromatids separation and nuclear division[95-98]. The transition from metaphase to anaphase is a highly regulated process for cells and depends upon the activation of the APC/C (Figure I5).

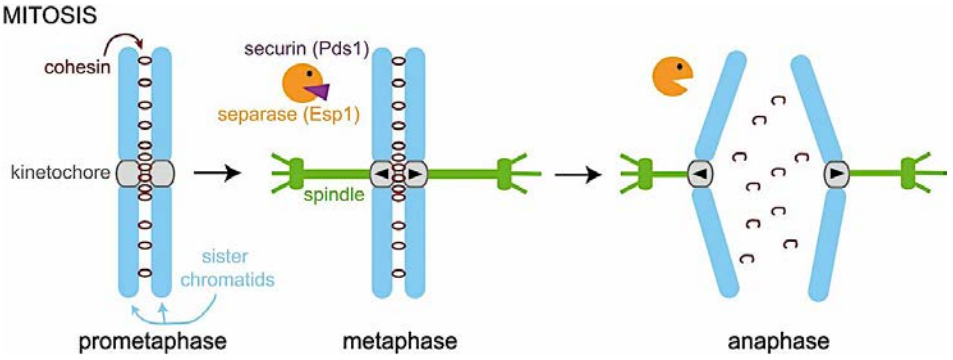

Figure I5. Chromosomes segregation during mitosis[99].
Schematic diagram showing the key features of chromosome segregation during *S. cerevisiae* mitosis.

In particular, Pds1 degradation is due to the APC/C^{Cdc20}–dependent ubiquitination, which leads to the activation of the proteolytic activity of Esp1 triggering entry into anaphase[95,100-106]. In this phase, once Scc1 is cleaved, the pulling forces arising only from the SPBs move each sister chromatid to one of the two

opposites poles of the cell, dividing the DNA content into two equal parts[94].

Interestingly, Scc1 cleavage alone is not sufficient to ensure mitotic exit. Indeed, two other conditions need to be satisfied in cells: degradation of mitotic cyclins and activation of the Cdc14 phosphatase. To obtain this, the assembly of the complete APC/C^{Cdc20} ubiquitin-ligase complex triggers the ubiquitination and proteasome degradation of many mitotic targets, such as CLB cyclins, recognized through their destruction box consensus[107-115].

Secondly, mitotic exit also requires the dephosphorylation of key CDKs substrates, promoted by Cdc14. This phosphatase is bound to an inhibitor, Net1, and kept inactive in the nucleolus for most of the cell cycle[46,116-118]. However, from early anaphase until telophase, the interaction between the two proteins is lost and Cdc14 spreads out in the cytoplasm, becoming active[116,119]. In *S. cerevisiae,* two different pathways allow the release and activation of Cdc14 triggering mitotic exit: the FEAR (cdc fourteen early anaphase release) and the MEN (mitotic exit network) (Figure I6).

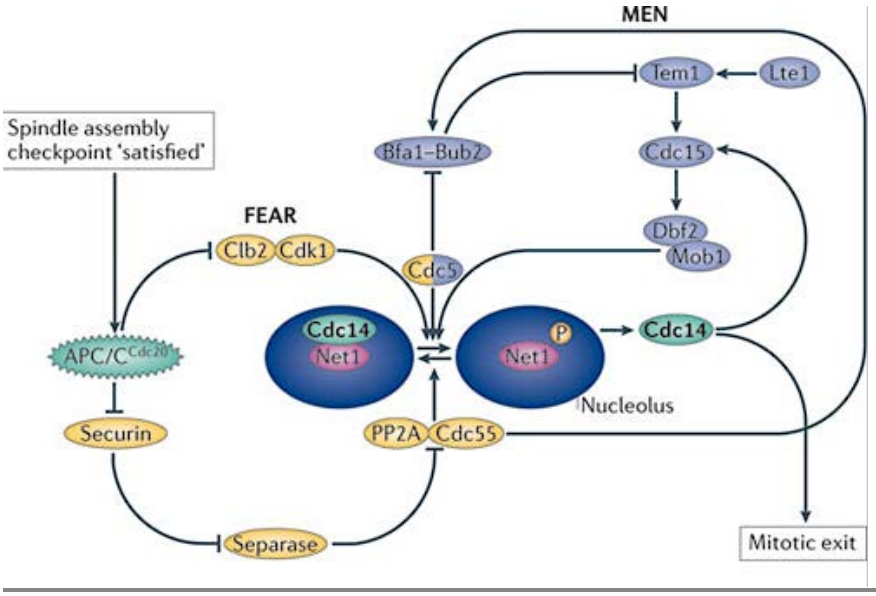

Figure I6. Regulation of Cdc14 activity during mitotic exit in budding yeast[117].

In budding yeast mitotic exit is ensured by the activation of Cdc14 through two different regulatory networks: the early anaphase release (FEAR; yellow) and the mitotic exit network (MEN; light blue).

8. Mitotic exit pathways in budding yeast

S. Cerevisiae has been widely used to study mitotic exit processes, which are guaranteed by the activity of Cdc14 phosphatase. Cdc14 function is to counteract CDK1 mitotic activity by activating through dephosphorylation three subrtates: the CDK1 inhibitor Sic1, a Sic1 transcription factor called Swi5 and the Cdh1 specificity factor for the APC/C complex. The APC^{Cdh1} targets key mitotic players for degradation, including CLB cyclins and Cdc5, allowing mitotic exit[116,118] .

As previously discussed, Cdc14 is kept inactive throughout most of the cell cycle, from G1 to metaphase anchored in the nucleolus by tight binding to its Net1/Cfi1 inhibitor in a nucleolar complex called RENT (Regulator of Nucleolar silencing and Telophase exit)[119,120]. This localization is strictly maintained in case of spindle mispositioning, preventing mitotic exit[121,122]. In particular, the timing of Cdc14 activation is a critical event for budding yeast cell cycle, since it has to take place only in early anaphase to support spindle elongation and in late anaphase to ensure that mitotic exit can be coupled with a successful chromosome segregation[123-125].

8.1 The FEAR (Cdc fourteen early anaphase release)

The FEAR (Cdc fourteen early anaphase release) pathway promotes the release of Cdc14 in the nucleoplasm at the metaphase to anaphase transition, but is not sufficient to promote CLB cyclins-CDK inactivation and exit from mitosis[126,127]. Conversely, the MEN (mitotic exit network) pathway drives Cdc14 full release into the cytoplasm later in anaphase, allowing its phosphatase activity on proper targets[128-130].

The FEAR network is composed by the separase Esp1, the polo-like kinase Cdc5, the kinetochore protein Slk19, the small nuclear protein Spo12 and the replication fork block protein Fob1[131-134].

CDK1 activity is necessary for the FEAR to release Cdc14, since it phosphorylates Net1, eliminating its interaction with Cdc14[135]. The FEAR is negatively regulated by the $PP2A^{Cdc55}$ phosphatase, which removes the phosphorylation performed by CDK1 and Cdc5 on Net1/Cfi1. $PP2A^{Cdc55}$ activity is inhibited by an additional

non-proteolytic activity of Esp1, which acts together with Slk19, Zds1 and Zds2 proteins[136-143].

Once released by FEAR, Cdc14 directs the establishment of a mother-directed pulling force that, together with the daughter-directed pulling force and elongating spindle, faithfully segregates the genetic material[127]. Moreover Cdc14 has, at least in part, a role at the onset of anaphase, when microtubule dynamics decreases dramatically, allowing the stabilization of the anaphase spindle and chromosome segregation. Indeed, Cdc14 dephosphorylates a number of microtubule-binding proteins, allowing them to interact with the elongating spindle and stabilize it[144,145]. The release of Cdc14 by the FEAR pathway is only transient and the phosphatase is then sequestered again in the nucleolus, until the activation of the MEN. Despite this, the Cdc14 FEAR-dependent partial release is able to ensure that segregation of all chromosomes happens at the same time[146].

8.2 The MEN (mitotic exit network)

The MEN is an essential pathway that promotes the release of Cdc14 in the cytoplasm at the end of anaphase[147-149]. MEN resembles a Ras-like GTPase signaling cascade and is composed by the GTPase Tem1, the bud-cortex protein Lte1, the GAP (GTPase activating protein) Bub2 in complex with Bfa1 and the protein kinases Cdc5, Cdc15, and Dbf2[150-153]. Tem1 is a G-protein that acts at the top of the MEN pathway, and its localization is cell cycle-regulated[120,154]. Indeed the protein localizes on the SPB during G1, on both SPBs after SPB-

duplication, and moves specifically on the daughter-directed SPB at the beginning of anaphase[155,156]. The activity of Tem1 is regulated by the Spindle POsitioning Checkpoint (SPOC, discussed below), which prevents mitotic exit if the spindle is not correctly oriented along the mother-bud axis[157]. If the SPOC is activated, the GAP complex Bub2-Bfa1 negatively regulates the GTPase activity of Tem1 and the cell can not exit from mitosis[158]. Once the SPOC is turned off, activation of Tem1 propagates the signal to the kinase Cdc15, which phosphorylates the Dbf2–Mob1 complex activating Dbf2[159]. This kinase then phosphorylates Cdc14 on serine and threonine residues adjacent to a nuclear-localization signal (NLS), thereby abrogating its NLS activity and promoting its transfer to the cytoplasm[160-163]. The exit of Cdc14 from the nucleus leads to the switch-off of mitotic CLBs-Cdc28 complexes and to mitotic exit[121, 164].

9. Spindle checkpoints

S. cerevisiae cell division is a strictly regulated process that allows the correct translocation of the spindle and a portion of the nucleus from the mother cell into the bud. During this process the spindle has to orient along the mother-bud axis, and then it is pulled into the bud. These events need to be properly accomplished to allow the cell to go through cytokinesis. Two pathways operate to prevent errors in spindle formation and orientation: the Spindle Assembly Checkpoint (SAC) and the Spindle Positioning Checkpoint (SPOC).

9.1 The Spindle Assembly Checkpoint (SAC)

The SAC pathway detects the lack of attachment of microtubules to kinetochores and arrests cell cycle progression to prevent errors during sister chromatids separation, avoiding chromosome missegregation and aneuploidy[165,166] (Figure I7).

There are two hypotheses on the possible mode of SAC action. The first is that the checkpoint recognizes the lack of microtubule attachment to the kinetochore, the second is that the checkpoint senses the absence of tension generated on the kinetochore by microtubules. Controversial data and the interdependence between microtubule attachment and tension make the comprehension of this mechanism still unclear[169-171]. The current model predicts that when microtubule-kinetochore attachments are not properly set up, the Aurora B kinase (Ipl1 in *S. cerevisiae*) promotes the turnover of connections between kinetochores and SPBs, creating unattached kinetochores that activate the SAC[169,170].

SAC network is composed by a set of conserved proteins: Mad1, Mad2, Mad3, Mps1, Bub1 and Bub3. These proteins accumulate on the outer side of unattached kinetochores, generating an inhibitory signal for the mitotic progression. When the checkpoint is activated, Mad2, Mad3 and Bub3 interact to form the Mitosis Checkpoint Complex (MCC)[172-175]. In particular, MCC function is to bind Cdc20, blocking its interaction with APC/C and preventing ubiquitinination of the Pds1 securin and anaphase onset.

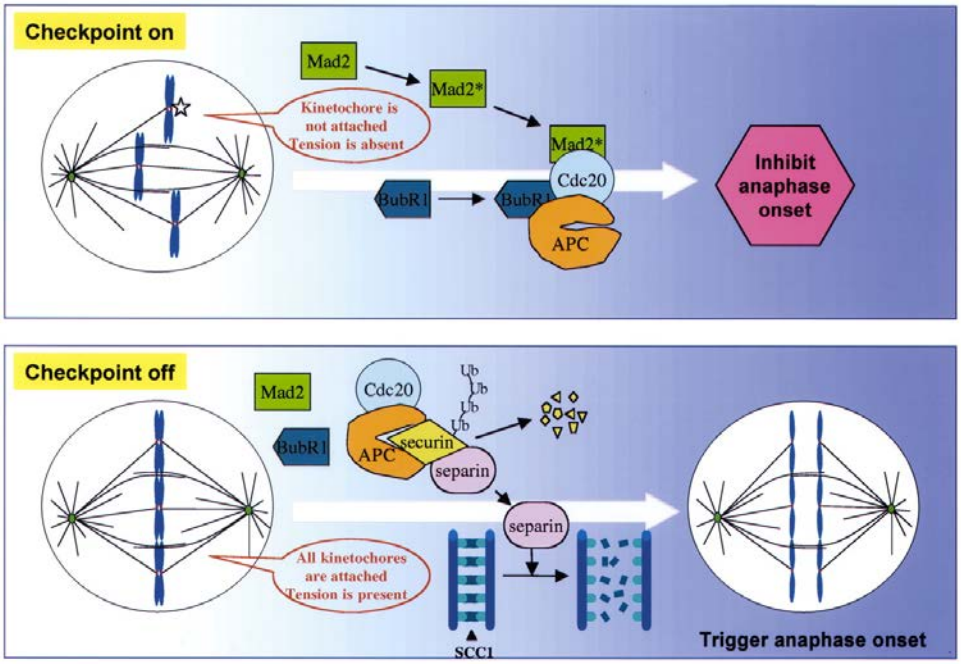

Figure I7. Schematic view of spindle assembly checkpoint (SAC) signaling[167].

Improperly attached kinetochores induce the SAC activation (upper panel). Once the checkpoint is on, the Mitosis Checkpoint Complex (MCC), which includes activated Mad2 (Mad2*) and BubR1, inhibits APC/C^{Cdc20} activity and anaphase onset. Once chromosomes are properly attached, the SAC is turned off, resulting in the activation of APC/C^{Cdc20}, securin degradation (Pds1 in budding yeast) and Scc1 cleavage by released separase (Esp1 in budding yeast; bottom panel).

Once microtubule-kinetochore attachment is correctly satisfied the SAC is switched off, triggering Pds1 ubiquitination by APC/C^{Cdc20}. The ubiquitinated form of Pds1 is degradated by the proteasome, triggering the separin Esp1 release. Free Esp1 is able to cleave

the Scc1 subunit of the sister-chromatid cohesion complex, event that, together with the degradation of CLB cyclins, ensures the separation of sister chromatids and the onset of anaphase[176-180]. The other components of the SAC Mad1, Bub1 and Mps1, amplify the SAC signal and the rate of MCC formation, recruiting more SAC proteins to the kinetochore[181]. All these events prolong pro-metaphase until all chromosomes are bi-oriented between the two poles of the spindle on the metaphase plate, effectively causing a temporary arrest at the metaphase to anaphase transition.

9.2 The Spindle Positioning Checkpoint (SPOC)

The SPOC is a network that, in case of incorrect spindle orientation, transiently arrests mitotic exit through MEN inhibition, delaying cytokinesis until the spindle is not oriented along the mother-bud axis to ensure that both cells receive one nucleus[182,183] (Figure I8).

The SPOC arrest relies upon inhibition of the small GTPase Tem1 by the GTPase-activating protein (GAP) complex Bub2-Bfa1. Bub2-Bfa1 inhibits Tem1 activity by stimulating GTP hydrolysis, thus keeping it in its GDP-bound inactive state[184,185]. When the spindle is misoriented and both SBPs are within the mother cell, the Kin4 kinase, associated to the SPBs, phosphorylates the Bub2-Bfa1 complex, preventing an inhibitory phosphorylation by Cdc5 and inactivating Tem1[158, 185-187].

If the spindle is correctly oriented and the daughter-directed SPB (dSPB) passes through the bud-neck, Lte1, a protein localized in the bud, disrupts Kin4 interaction with the dSPB and allows Cdc5 to phosphorylate Bub2-Bfa1. This inhibitory phosphorylation allows Tem1 activation, which triggers MEN, mitotic exit and cytokinesis[188-190].

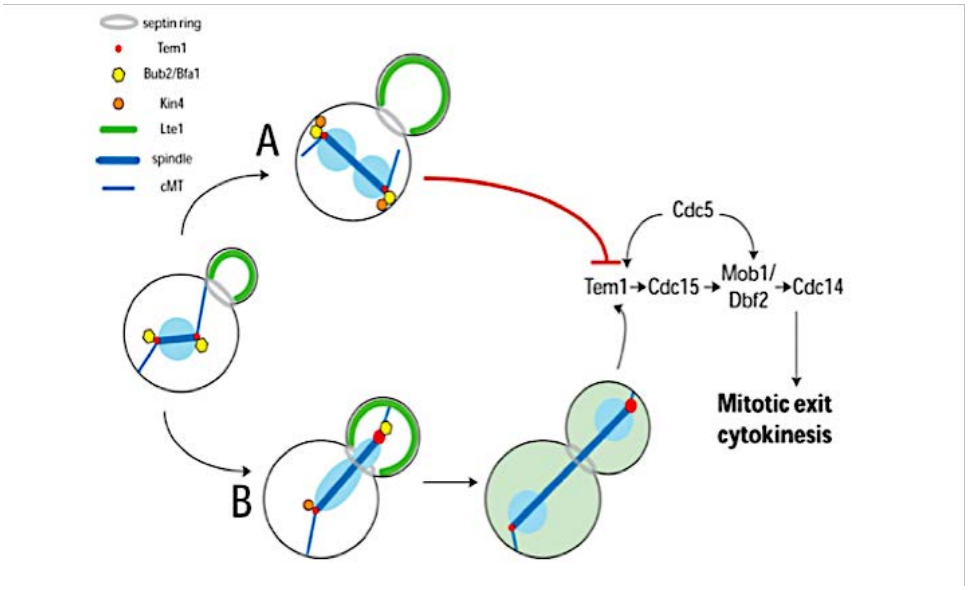

Figure I8. The Spindle POsitioning Checkpoint (SPOC)[182].
The SPOC is activated in response to spindle misalignment (A) and is switched off when the spindle is properly oriented along the mother-bud axis (B).

10. Polarized growth in *S. cerevisiae*

Polarization is essential for cells morphogenesis, differentiation and proliferation. During the phase of polarization, cell growth and

material deposition are directed towards specific areas of the cell periphery. It has been shown that the actin-rich cell cortex of several eukaryotic cells responds to spatial cues and provides the machinery that polarizes the cell. Studies in budding yeast extend this view. Rho GTPases and cyclin-dependent protein kinases coordinately regulate the polarization mechanism in *S. cerevisiae*[191-193].

Polarized growth in budding yeast goes through two different steps during the cell cycle: the first is the apical growth (from START to the begin of M-phase) that allows cells to grow asymmetrically and produce a bud; the second is the isotropic growth that consists in an uniform size increase of mother and daughter cells (during M and early G1).

The key factor of *S. cerevisiae* polarization is actin, a globular multi-functional protein that forms microfilaments and has been found in all eukaryotic cells. Actin guides growth by directing the delivery of internal membranes and other factors[194,195.] During polarized growth actin accumulates first at the presumptive bud site and then at the bud tip, while during isotropic growth actin is redistributed all over the bud surface and actin cables extend from the mother cell into the bud forming a network[196,197].

Yeast cells contain three types of actin structures: actin cables, actin cortical patches and an actin-myosin contractile ring. Actin cables constitute tracks for mitotic spindle alignment, polarized secretion and organelle transport. Cortical patches are branched

actin filaments involved in endocytosis, membrane growth and polarity. Lastly, the actin-myosin ring gets assembled at the mother-bud neck to ensure cytokinesis[26, 196, 198].

10.1 Cdc42

Polarization characterizes the growth of cells of almost all living organisms. Cdc42 is a small Rho like GTPase that plays the major role in regulating polarized growth of budding yeast, such as from many others eukaryotes to human cells. Rho GTPases are conserved structurally and functionally by evolution, acting in diverse organisms as key signaling molecules in polarity control[199-202].

Through its interactions with a variety of downstream effectors (e.g. Bnr1, Bni1, Bud6, Ste20, Cla4 and Gic2) Cdc42 modulates cell polarization in different cellular processes. It promotes budding or mating, localizing at the presumptive bud site or mating projection and triggering actin cytoskeleton assembly and targeted secretion; regulates actin cables nucleation and localization through the formins Bni1 and Bnr1; is involved in vesicles transport and septin ring deposition[203-205] (Figure I9).

Cdc42 localizes at this presumptive bud site in late G1 and to sites of polarized growth during the rest of the cell cycle, where it activates effectors that signal to actin cytoskeleton[206,207] (Figure I9). A temperature sensitive (Ts) mutant *cdc42-1* fails to bud at 37°C and shows a random distribution of actin, indicating that Cdc42 is essential for polarized organization of the actin

cytoskeleton[202].

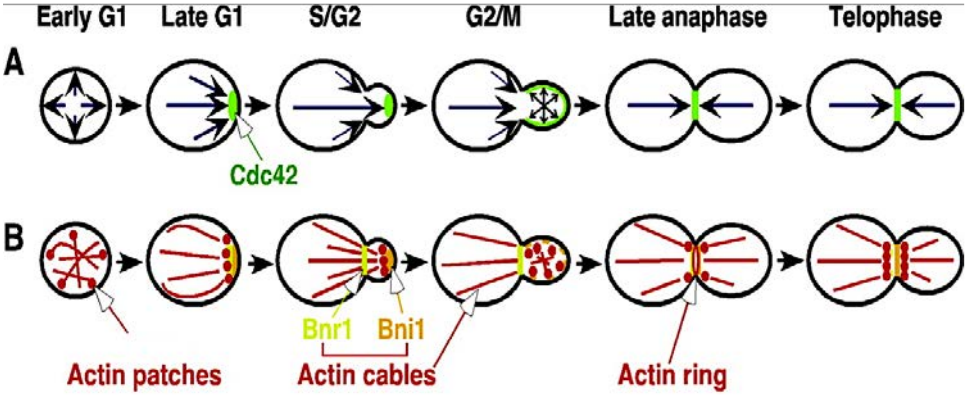

Figure I9. Localization of Cdc42, actin and formins during the cell cycle[200].

(A) Cdc42 localization (green) and the directionality of cell growth (blue arrows) in the cell cycle. (B) Actin patches (red spots), actin cables (red lines), the actin ring (red circle) and the localization of formins Bni1 (brown) and Bnr1 (yellow) are indicated.

In a still unclear mechanism Cdc42 activation leads to polarization of the actin cytoskeleton, assembly of septin filaments, and polarization of various cortical proteins[193]. Polarization process starts in G1 with Cdc42 accumulation at the "prebud site" from which the bud will emerge. After bud emergence Cdc42 remains active at the bud tip, where it directs growth of the daughter cell manipulating actin organization. At the end of mitosis, Cdc42 activity decreases to allow cytokinesis.

As other GTPases, Cdc42 can switch between a GTP-bound active conformation, which allows it to bind to different effectors,

and a GDP-bound form that is inactive[208]. GAPs (GTPase activating proteins) stimulate the conversion of GTP to GDP, inactivating Cdc42. Budding yeast contains four GAPs for Cdc42: Bem2, Bem3, Rga1 and Rga2[209]. Bem2 and Bem3 act during bud site formation, while Rga1 and Rga2 operate during septin ring formation and in response to mating pheromone[210,211]. Guanine Exchange Factors (GEFs) contrast the inhibitory effect of GAPs on GTPases, promoting the substitution of GDP with a GTP[212].

The only and essential GEF of Cdc42 is Cdc24, which is required for bud emergence and establishment of cell polarity[211,213]. Coversely, GDI (guanine-nucleotide dissociation inhibitors) prevent the dissociation of GDP from Rho proteins, keeping them in the inactive state[214]. Budding yeast contains only a GDI, Rdi1, which modulates Cdc42 activity and localization[215-217].

10.2 Ras

Ras proteins are GTPases that act in the switching of pathways regulating cell growth and differentiation in eukaryotic cells, resulting fundamental in cell cycle regulation. Their activity is influenced by the cycle between active GTP-bound and inactive GDP-bound forms[218-220]. *S. cerevisiae* genome encodes for two homologues of Ras: *RAS1* and *RAS2*. Moreover, this organism presents two GEFs for Ras, the essential Cdc25 and the dispensable Sdc25, and two GAPs, Ira1 and Ira2[221-233]. The main role of Ras1 and Ras2 in *S. cerevisiae* is to promote Cyr1 activity, the adenylate cyclase (AC) responsible for cAMP production[234-

[236]. Once in the GTP-bound form Ras induces Cyr1-dependent synthesis of cAMP, which releases the Protein Kinase A (PKA) complex from its inhibitory subunit Bcy1, promoting cell cycle progression[237]. In particular, PKA is an heterotetrameric complex containing two catalytic subunits and two regulatory subunits: *TPK1*, *TPK2* and *TPK3* encode isoforms of the PKA catalytic subunits, conversely *BCY1* encodes for the regulatory subunit. In absence of cAMP two Bcy1 regulatory subunits mask the catalitic ones, keeping PKA inactive. Binding of cAMP causes the dissociation of the Bcy1 regulatory subunits, inducing PKA activation[238,239]. Once activated by Ras through cAMP production, PKA is able to influence cellular growth acting on post-translational modifications of its targets and altering gene expression (Figure I10).

In response to external factors, active PKA is able to commit cell cycle begin through START, which is usually inhibited by Whi3, a RNA-binding protein that sequesters Cln3 mRNA in cytoplasmic foci, preventing nuclear accumulation of Cdc28-CLNs complexes[240,241]. In particular, PKA inhibits Whi3 through a phosphorylation on S568, inducing an increase in Cln3 levels that triggers degradation of the CKIs[242].

Ras activity relies on proper localization on the plasma membrane (PM), achieved after a series of irreversible farnesylation of its terminal CAAX motif, followed by reversible palmitoylation by the Erf2/Erf4 complex[244,245.] Despite the actual model for Ras recruitment at the PM is still not completely clarified, both

secretory pathways and activity of Erf2/Erf4 complex result involved[244,246,247].

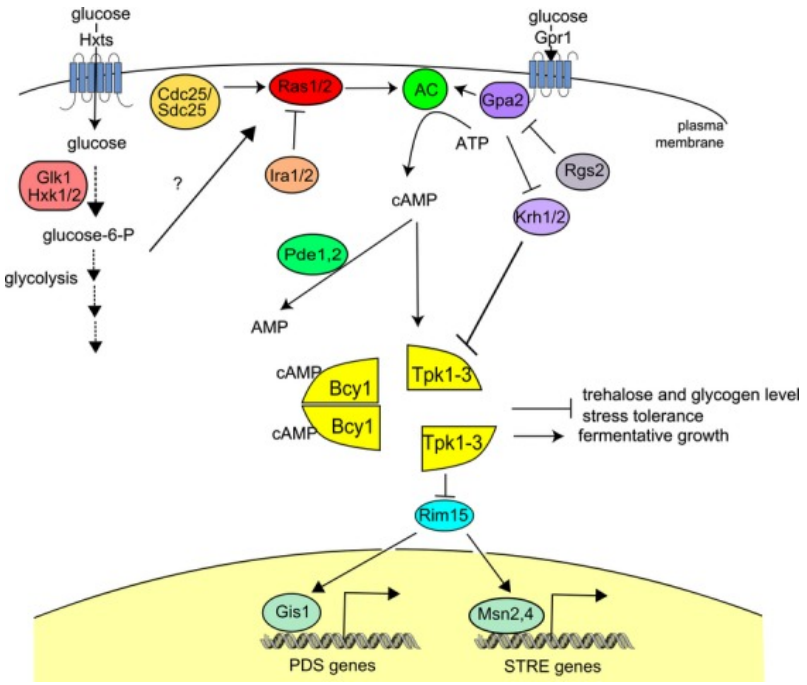

Figure I10. Glucose activation of the cAMP-PKA pathway [243].

In *S. cerevisiae* the Cdc25, Sdc25-Ras1,2-Ira1,Ira2 system senses intracellular glucose through glucose catabolism in glycolysis in a pathway that is still not totally clarified (in orange/red). cAMP binds to the Bcy1 regulatory subunits of PKA causing dissociation and activation of the catalytic subunits, Tpk1-3 (in yellow).

Since Cdc25, Ira1 and Ira2 are mainly localized to ER and mitochondria, respectively, it is possible that Ras regulation occurs before its plasma membrane (PM) accumulation, providing

an alternative level of modulation of this pathway through spatial regulation of its players[248]. Although a remarkable lack of knowledge about Ras activity in mitosis needs to be filled, works in different organisms reported a physical interaction between Cdc24 and Ras[249].

Ras structure is highly conserved, together with its GEFs and GAPs, whose catalytic domains are found with high homology in many eukaryotic organisms[221,222]. Nevethless Ras effectors are profoundly different in budding yeast and higher eukaryotes (PKA pathway in *S. cerevisiae* and MAPKs in high eukaryotes), the molecular mechanisms of Ras regulation are conserved by evolution[250]. For instance, Ras strong conservation is justified by its fundamental role in cell cycle regulatory pathways. In higher eucaryotes indeed, alteration of Ras network often characterizes many types of human cancers[251,252].

11. Haspin

Haspin (*ha*ploid germ cell-*sp*ecific nuclear prote*in* kinase) is a nuclear atypical serine/threonine kinase first identified in mouse testis cells[253]. Haspin C-terminal domain matches significantly to the sequence of many eukaryotic protein kinases. The rest of haspin sequence, however, contains distinctive inserted regions and lacks of a subset of conserved residues, present in several known kinases. For these features haspin-like proteins have been classified as a novel eukaryotic kinase family. Proteins sharing similarity with haspin have been identified in several eukaryotes,

including yeasts, plants, flies, fishes, and mammals[254]. Structure can change from one organism to the other, but the kinase domain, the leucine-zipper and several putative phosphorylation sites result often conserved by evolution (Figure I11)[253,255]. In higher eukaryotes haspin gene is located inside the intron of integrin alphaE, and is characterized by total lack of introns and some transposon-like features[256].

In mammalian cells haspin phosphorylates H3-T3 during metaphase, promoting, together with phosphorylated histone H2A-S121, the recruitment of the chromosomal passenger complex (CPC)[257-259]. In the absence of hasipin-dependent H3-T3 modification, cells arrest in mitosis and proper chromosome positioning at the metaphase plate is prevented[260,261]. Moreover, it has been shown that Aurora B, the catalytic subunit of the CPC complex, phosphorylates haspin promoting its recruitment at inner centromeres in mitosis[262]. Haspin regulation plays on two different levels: localization and post-translational modifications.

Firstly haspin localization changes during the cell cycle: the protein is already in the nucleus during interphase, but it is not bound to chromosomes and has no access to its chromatinic substrates[261,263]. To ensure haspin chromatinic recruitment both CDK1 priming phoshporylation on T206 and the involvment of SUMOylated topoisomerase IIα are required[264,265].

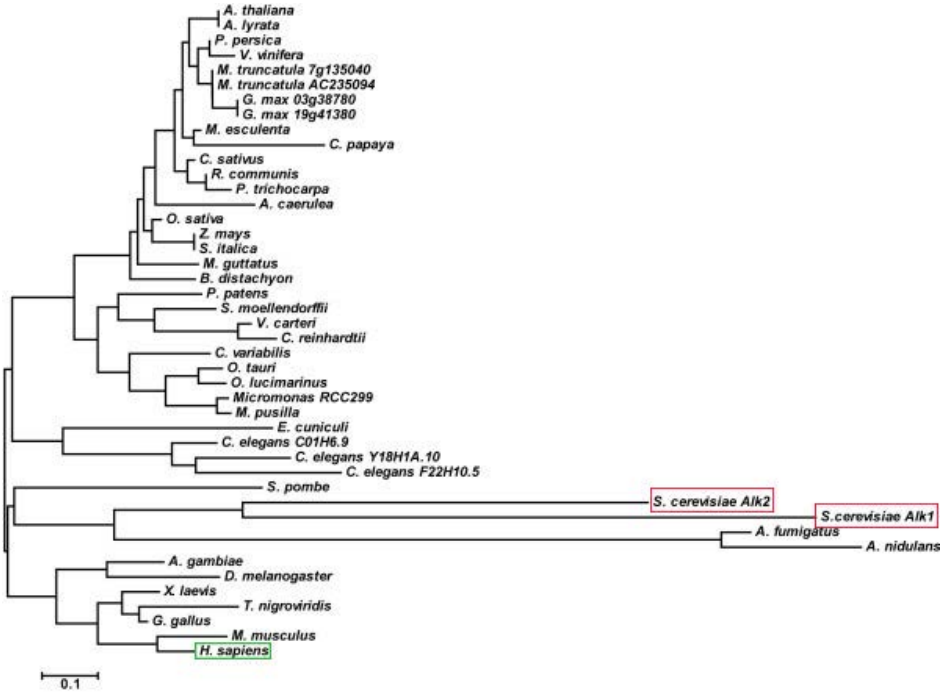

Figure I11. Multiple alignment of Haspin kinases in the kinase domain[255].

Haspin kinase domain from different species have been aligned. Red boxes indicate *S. cerevisiae* Alk1 and Alk2; green box indicates human haspin.

The second level of haspin regulation is guaranted by the presence of a conserved basic region, which induces haspin autoinhibition during interphase. This autohinibitory domain is folded onto the catalytic domain, preventing misregulated haspin activity[266]. Haspin autoinhibition is neutralized when CDK1 phosphorylates its N-terminus, recruiting the Plk1 Polo-like kinase, which, in turn, further phosphorylates multiple sites at the haspin N-terminus. These Plk1- dependent modifications activate

haspin, resulting in the phosphorylation of H3-T3[263,266,267] (Figure I12).

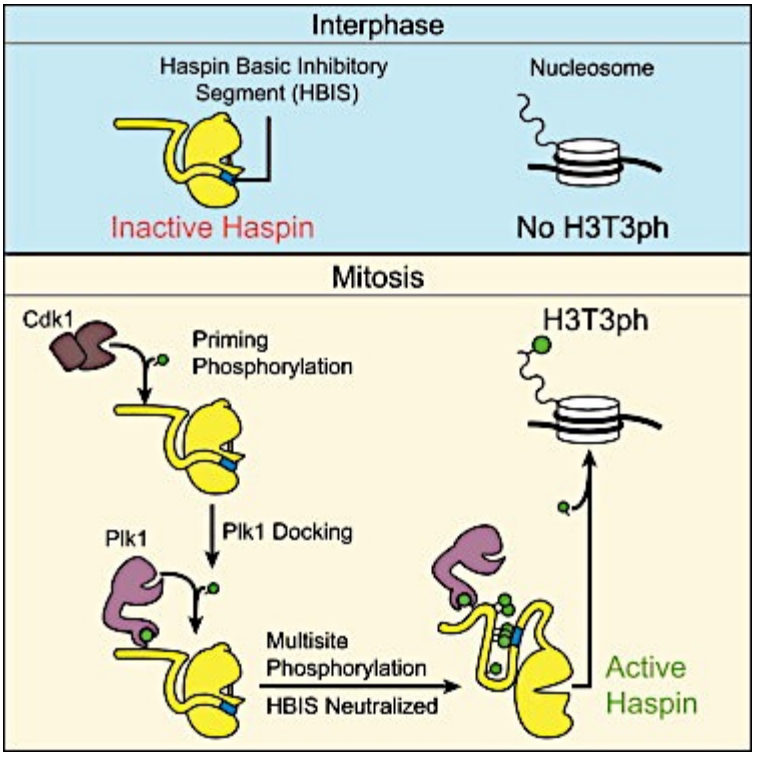

Figure I12. Autoinhibition and Polo-dependent multisite phosphorylation restrict haspin activity to mitosis[266].

During interphase a conserved basic segment autoinhibits haspin (upper panel). This autoinhibition is neutralized in mitosis when Cdk1 phosphorylates haspin in order to recruit Polo-like kinase (Plk1), which further phosphorylates multiple sites at the Haspin N-terminus (lower panel).

Haspin signaling through H3-T3 phoshporylation seems involved not only in chromosome segregation, but also in stem cells

identity maintenance through multiple divisions. In particular, studies in *Drosophila* demonstrate that pre-existing and newly synthesized histone H3 are asymmetrically distributed during *Drosophila* male germline stem cell (GSC) asymmetric division[268]. Recent observations demonstrate that haspin-dependent H3-T3 phosphorylation is needed to distinghuish pre-existing versus newly synthesized H3, coordinating asymmetric segregation of "old" H3 into GSCs[269].

Haspin H3-T3 activity was also reported in fission yeast and in *Arabidopsis thaliana*, where the kinase is involved in plant development during embryonic patterning[270, 271].

In *Saccharomyces cerevisiae,* it was reported that haspin deletion is not synthetically lethal with the *sli15(ΔNT)* mutation, which leads to premature recruitment of Ipl1 to the spindle. This observation was not sufficient to demonstrate a possible involvement of hapin in the Aurora B pathway[272]. However recently, it was published that topoisomerase IIα is required for recruitment of the tension checkpoint kinase Ipl1/Aurora B to inner centromeres in metaphase. Genetic and biochemical evidences suggest that topoisomerase IIα recruits Ipl1 via the haspin–H3-T3 phosphorylation pathway[264]. By the use of *S. cerevisiae* as model system to study new pathways in which haspin can be involved, our group found that it regulates polarity cues necessary for mitotic spindle positioning[273].

Budding yeast contains two haspin paralogues: *ALK1* and *ALK2. ALK1* was initially identified in 1998, during a study on cell cycle-regulated genes of *S. cerevisiae*, as a member of the *CLB2* gene cluster, while *ALK2* was later identified as *ALK1* homologue[256,274,275].

Analyses in synchronous yeast cultures demonstrated that Alk1 and Alk2 are phosphorylated during mitosis and that their levels oscillate throughout the cell cycle, peaking in M and S/G2-phase, respectively. In addition both proteins contain a D-box and a KEN-box, which are typical for proteins whose level is controlled by APC/C, corroborating the hypothesis of a cell cycle dependent regulation on the proteins stability[276-278].

Phenotypic analyses demonstrated that *alk1Δalk2Δ* cells are extremely sensitive to microtubule depolymerizing drugs, such as nocodazole or benomyl. This sensitivity is due to an abnormal distribution of several polarization factors that, in conditions that delay the mitotic progression, severely compromise cells vitality[273]. In fact, after mitotic delay, *alk1*Δ*alk2*Δ cells show a misdistribution of actin and compromised localization of formins and other polarity factors. In *alk1*Δ*alk2*Δ cells entering in M-phase, actin accumulates mainly into the bud and is not redistributed equally between mother and daughter[195,273]. The Bnr1 formin, which is usually found only at the bud-neck, in haspin-defective cells accumulates also at the bud tip[273]. This mislocalization influences also other factors involved in yeast cell polarity, such as the polarisome component Bud6, whose localization at the

bud-neck is lost in haspin-deficient cells[273].

All these phenotypes lead *alk1*Δ*alk2*Δ cells to accumulate an excessive force driving polarity toward the bud, causing the spindle to misposition and to elongate only in the bud, resulting, after nuclear division, in an anucleated mother and a binucleated daughter, both of which will not generate a live cell population[273].

AIM OF THE PROJECT

This work aims at identifying the physiological functions of haspin kinase and define the corresponding molecular mechanisms. These studies resulted in two manuscripts (one in preparation and one in submission) focused on haspin kinase involvement in controlling the G2/M transition and on how haspin modulates the dispersion of the polarisome in mitosis, respectively.

We previously showed in budding yeast that haspin is needed for the regulation of mitotic spindle positioning and for the tolerance of mitotic delays. We proceeded to further explore haspin functions by utilizing two different strategies. First, we tried to unravel the molecular mechanism involving haspin and its targets in polarization. Interestingly, we found that the role of haspin in polarization relies on its ability to modulate Ras localization. Our findings suggest a new mitotic role for Ras that, by regulating Cdc24 redistribution, impacts on Cdc42 GTPase activation at polarized sites. Since alterations in cell polarization are characteristics of cancer cells, these observations may help in the comprehension of molecular basis of cancer development.

We also looked for new haspin functions in yeast. We unexpectedly identified an involvement of haspin orthologue Alk1, but not of the Alk2 paralogue, in controlling the inhibitory tyrosine phosphorylation on CDK1 at the G2/M transition. Cell cycle misregulation is one of the most evident hallmarks of cancer cells. Cells rely on surveillance mechanisms, named checkpoints, to

arrest cell cycle progression in response to stress conditions and to promote restoration of normal conditions; this is essential to preserve genomic and cellular integrity. Several alterations activate Wee1-like kinases in G2, inhibiting CDK1 and delaying entry into mitosis. When cells are ready to proceed further in the cell cycle, Cdc25-like phosphatases dephosphorylate CDK1 at specific sites, allowing cell-cycle progression. In budding yeast we observed that deletion of the *ALK1* haspin orthologue reduces the inhibitory phosphorylation on CDK1. We therefore investigated the involvement of haspin in the balance between Swe1 and Mih1 activities, the yeast orthologous of Wee1 and Cdc25 respectively. In particular, we demonstrated that Alk1 influences Mih1 phosphatase activity. Despite this pathway has a different function in *S. cerevisiae* respect to human cells, the molecular mechanisms of this regulation may be conserved from yeast to mammals.

MAIN RESULTS

In this section I will recapitulate the main results presented:

1. In the Draft Manuscript (attached in Part II):

Martina Galli, Roberto Quadri, Elena Galati, Davide Panigada, Laura Diani, Paolo Plevani, Marco Muzi-Falconi

"The *S. cerevisiae* Alk1 haspin orthologue regulates the G2/M transition in response to morphogenetic stress"

2. In the Submitted Manuscript to Nature Communications (attached in Part III):

Roberto Quadri, **Martina Galli,** Elena Galati, Giuseppe Rotondo, Guido Roberto Gallo, Davide Panigada, Paolo Plevani, Marco Muzi-Falconi

"Haspin regulates Ras localization to promote mitotic Cdc24-driven depolarization"

Observations obtained in our laboratory report that loss of budding yeast haspin paralogues Alk1 and Alk2 confers sensitivity to M-phase delays[273]. In particular, *alk1*Δ*alk2*Δ cells lethality is due to the hyper-polarization of actin and polarity factors toward the bud, which results in the missegregation of both nuclei in the daughter cell.

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1. Haspin regulates the G2/M transition of *S. cerevisiae* in response to morphogenetic stress

Our previous data revealed that *S. cerevisiae* is a good model organism to study haspin function beyond the well-established H3-T3 phosphorylation. In order to explore new functions for haspin kinase in cell cycle regulation, we started identifying possible upstream regulators for Alk1 and Alk2 proteins. In this context we chanced upon Elm1, a kinase required for efficient cytokinesis and regulation of Swe1, the key player of the morphogenesis checkpoint. In particular, a preliminary observation showed that phosphorylation of Alk1 is abolished in *elm1*Δ cells**.** Since this data pointed toward an involvement of Alk1 in the morphogenesis checkpoint, we decided to induce actin alterations to study checkpoint activation in strains lacking for haspin activity. Firstly, we observed that deletion of *ALK1* causes a defect in the response to Latrunculin A induced morphogenetic stress. Secondly, we found that cells lacking *ALK1* fail to properly

arrest cell cycle progression at G2/M even upon misregulation of Cdc42, obtained by the use of a *cdc24-1* defective allele. Consistently with this, we also observed that the Cdc28-Y19 phosphorylation signal decreases significantly in *alk1*Δ cells during the morphogenetic stress. In order to discriminate if this defect was due to a misregulation of Swe1 or Mih1 checkpoint regulators, we decided to combine *ALK1* and *MIH1* deletions. Interestingly, while *ALK1* deletion reduces Cdc28-Y19 phosphorylation, additional removal of *MIH1* partially restores a wild-type level for this modification, toghether with a correct pattern of nuclear division. Taking these observations into accout, we concluded that loss of *ALK1* causes a precoscious Mih1 activation.

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2. Haspin regulates Ras localization to promote Cdc24-dependent dispersion of polarity clusters

Starting from our observations on *alk1*Δ*alk2*Δ cells lethality following an M-phase delay, we decided to investigate further the cause for the hyper-polarization observed in *alk1*Δ*alk2*Δ cells. Firstly, we showed that the cause of actin and nuclear misdistribution of haspin depleted cells is due to the mislocalization of Bud6, which was lacking from the bud neck and hyper-accumulated at the bud tip. Then we demonstrated that Bud6 defect is caused by the misdistribution of Cdc42, the small

GTPase responsible for the polarization of eukaryotic cells. In particular, we found that, in cells lacking for haspin, the GTP-loaded form of Cdc42 is accumulated at the bud tip rather than being uniformly diffused on the plasma membrane. We reasoned that, since Cdc24 is the unique GEF in budding yeast for Cdc42, the most plausible explanation for GTP-loaded Cdc42 accumulation at the bud tip could be a similar misdistribution of Cdc24. By analysing Cdc24 localization in wild-type and *alk1*Δ*alk2*Δ cells during M-phase, we found that also Cdc42 GEF is accumulated at the bud tip, consistently with previous observations. Nevertheless Rsr1 is indicated in literature as responsible for Cdc24 accumulation at the plasma membrane, we observed that during mitosis its absence does not influence Cdc24 localization. Conversely during M-phase, we found that Cdc24 recruitment to the plasma membrane is dependent upon Ras, suggesting that Rsr1 role is required only in G1-phase. Finally, we discovered that the hyper-polarization characterizing haspin depleted cells during an M-phase delay is due to Ras and active Ras misaccumulation, constituting the cause for nuclear missegregation and cellular lethality.

CONCLUSIONS AND FUTURE PROSPECTS

Cell cycle misregulation is one of the most evident hallmarks of cancer cells. Cells rely on different checkpoints to arrest cell cycle progression at a specific phase in response to stress conditions, allowing time to repair and granting maintenance of genomic and cellular integrity. This thesis deepens our comprehension on haspin functions in budding yeast G2/M cell cycle switch. Our observations demonstrated that Alk1 haspin orthologue regulates budding yeast morphogenesis checkpoint, which controls the G2/M transition. Moreover, we found that Alk1 function is to inhibit Mih1 activity, one of the main regulators of the checkpoint. Despite interesting new results, the way haspin acts on G2/M transition still need to be clarified. In particular, we collected many genetic indications on Alk1 ability to influence Mih1 function, but the molecular mechanism underlying this regulation still needs to be elucidated. Moreover, another interesting point to be investigated will be Alk2 involvement in this network. Indeed, we found that *ALK2* deletion rescues the checkpoint defect of *alk1*Δ cells, raising the possibility that the two prologues have opposite roles in this regulation. Overall, our results extend the research field on haspin, revealing new pathways in which the kinase could be involved. Mih1 is conserved in higher eukaryotes where it is encoded by *CDC25*. Since Cdc25 regulates key transitions between cell cycle phases, it is not surprising that its misregulation has been reported in many human cancers, making

it a good target for anticancer therapies. Future studies will be focused in clarifying if loss of haspin activity could be responsible for Cdc25 misregulation also in human cells, leading to cell cycle alterations, genomic instability and cancer.

In the second part of this thesis we focused on haspin function in cell polarization. Polarization is a fundamental process for cellular development, proliferation and differentiation. One of the open questions in this field is how the redistribution of polarity factors occurs. Previously, we showed that in *S. cerevisiae* haspin is important for the tolerance of mitotic delays, regulating spindle positioning and nuclear segregation. Further investigating this new function for haspin, we found that the kinase is necessary in budding yeast for the polarisome dispersion. Moreover, we demonstrated that the functions in M-phase delay tolerance and polarisome dispersion are correlated. Indeed, in the second manuscript presented in this thesis, we showed that haspin function in polarisome dispersion relies on its ability to regulate Ras localization. In particular, we ascribed also a new function for Ras during M-phase in the activation of Cdc42 GTPase on the plasma membrane of the bud. These new findings deepen our knowledge on polarization, demonstrating that haspin regulates nuclear segregation together with polarity factors, which need to be properly redistributed during M-phase through Ras regulation on Cdc42.

Alterations in cellular polarization characterize often cancer cells, which by mutations modify their features and acquire the ability to

spread across the organism, becoming malignant. Therefore, also results presented in this second manuscript become fundamental to shed light on pathways necessary for cellular polarization and proliferation, which can easily result misregulated during carcinogenesis. In the future it will be interesting to carry on studies in this field, trying to reconfirm observations in budding yeast also in human cell lines and opening new possibilities in long term for cancer treatments focused on haspin and Ras possible misregulations.

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Part II

Manuscript I

Content: Draft Manuscript

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"The *S. cerevisiae* Alk1 haspin orthologue regulates the G2/M transition in response to morphogenetic stress"

The *S. cerevisiae* Alk1 haspin orthologue regulates the G2/M transition in response to morphogenetic stress.

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ABSTRACT

Haspin is an atypical protein kinase responsible for histone H3-T3 phosphorylation and for regulation of various aspects of cell cycle progression. In the yeast *Saccharomyces cerevisiae,* cells lacking *ALK1* haspin homologue fail to arrest the cell cycle in response to Latrunculin A treatment, and divide the nucleus in the absence of a bud. This phenotype is recapitulated in *alk1*Δ cells when the Cdc24 guanine nucleotide exchange factor of Cdc42 is inactivated. Deletion of *SWE1*, a CDK1-regulating kinase, inactivates the morphogenesis checkpoint and exhibits a phenotype very similar to that of *alk1*Δ cells, albeit more extreme. These observations indicate that Alk1 plays a critical role in the mechanism that modulates CDK1 activity in response to perturbation of actin cytoskeleton. Indeed, kinetic analyses following morphogenetic insults reveal that Cdc28-Y19

phosphorylation is activated but not maintained in the absence of *ALK1*. Although, *alk1*Δ cells do not exhibit altered levels of Swe1 or Mih1 proteins, we show that the reduced phosphorylation of Cdc28-Y19 is due to a precoscious activation of Mih1 when Alk1 is not functional. Intriguingly, this defect in morphogenesis checkpoint maintenance is rescued by the concomitant deletion of *ALK2*, a paralogue of *ALK1*, suggesting that the underlying mechanism is indeed quite complicate. Overall, the data presented in this work reveal a novel role for haspin kinase as critical for the G2/M cell cycle switch of *Saccharomyces cerevisiae*, in response to morphogenetic insults.

INTRODUCTION

Haspin is a serine/threonine atypical kinase that phosphorylates H3-T3 during metaphase, promoting the recruitment of the chromosomal passenger complex (CPC) at kinetochores[1-5]. A feedback loop, involving Aurora B - the catalytic subunit of the CPC - has been shown to promote haspin recruitment at inner centromeres in mitosis[6]. Haspin depletion in mammalian cells leads to cell arrest in mitosis and prevents proper chromosome positioning at the metaphase plate[7-10]. Haspin activity is cell cycle dependent: during interphase the protein is already in the nucleus, but a conserved basic region of haspin itself inhibits it. The autoinhibitory domain is folded onto the catalytic domain, preventing haspin activity[11]. Cyclin-dependent kinase 1 (CDK1)

neutralizes haspin autoinhibition with a phosphorylation at its N-terminus, followed by further phosphorylations at multiple sites performed by the Polo-like kinase 1 (Plk1). These Plk1-dependent modifications activate haspin, resulting in the phosphorylation of H3-T3[11,12]. Studies in *Drosophila* demonstrate that pre-existing and newly synthesized histone H3 are asymmetrically distributed during *Drosophila* male germline stem cell asymmetric division[13]. Recent observations show that haspin-dependent H3-T3 phosphorylation is necessary to distinguish pre-existing versus newly synthesized H3[14].

The *Saccharomyces cerevisiae* genome encodes for two haspin paralogues *ALK1* and *ALK2*[15]. Previous observations show that *ALK1* and *ALK2* deletion is not synthetically lethal with the *sli15(ΔNT)* mutations that leads to premature recruitment of Ipl1 to the spindle. These data were not conclusive to exclude a possible involvement of hapin in the Aurora B pathway[16]. However, it was recently published that topoisomerase IIα is required for recruitment Ipl1/Aurora B to inner centromeres in metaphase via the haspin–H3-T3 phosphorylation pathway[17].

ALK1 and *ALK2* genes code for two proteins whose levels peaks in mitosis and G2-phase respectively and that are phosphorylated during the cell cycle[15,18]. We previously reported that Alk1 and Alk2 are critical factors to coordinate polarization and cell cycle progression in *S. cerevisiae*, ensuring the correct positioning of several polarity factors following a transient mitotic delay[19]. These

findings suggest that budding yeast is a good model organism for identifying new processes where haspin kinase may be involved in regulating mitosis.

Mitotic entry is promoted by elevated CDK1 activity. At the G2/M transition, however, cyclin-dependent kinase 1 (CDK1) is inhibited by a phosphorylation on Y15 performed by Wee1-like kinases, which delays entry into mitosis[20-21]. Once all the conditions for G2/M transition are achieved, Cdc25-like phosphatases remove CDK1-Y15 inhibitory phosphorylation triggering CDK1-cyclins activation and mitotic entry[22-25].

In budding yeast, Wee1 kinase is encoded by the *SWE1* gene and modulates Cdc28 through an inhibitory phosphorylation on Y19. However, Cdc28-Y19 regulation does not seem to be involved in M-phase initiation in budding yeast. On the other hand, such regulatory circuit is essential for cell viability upon morphogenetic stress. This is confirmed by the observation that lack of Swe1 or Mih1 (the yeast orthologue of Cdc25 phosphatase) does not impair normal cell cycle progression in unperturbed conditions[26]. Swe1 activity on Cdc28-Y19 becomes fundamental upon insults to cytoskeletal structures in a pathway known as the “morphogenesis checkpoint”[27-33]. This surveillance mechanism couples bud formation to nuclear division by monitoring actin and septin organization, the presence of the bud and even its size. Any disruption or alteration of the actin cytoskeleton causes activation of this checkpoint, leading to a cell

cycle arrest at the G2/M transition with replicated DNA and Cdc28 phosphorylated on Y19, thus preventing nuclear division. Once cellular morphogenesis is restored, Swe1 is inactivated and degraded and the Mih1 phosphatase removes the Cdc28-Y19 modification, allowing completion of the cell cycle[34-41]. Cells deleted for *SWE1* cannot activate the checkpoint entering mitosis even in the absence of a bud; this results in nuclear division within a single cell compartment[27-33].

Most works have focused on the ability of the morphogenesis checkpoint to inhibit mitotic entry. However, activation of this process was also found to cause delays later during mitosis, primarily in metaphase, through inibhition of APC/C activity[42-47]. Moreover, evidences in budding yeast show that deletion of *MIH1* induces only mild delays in mitotic entry and anaphase onset, suggesting the possible contribution of other phosphatases[47-50]. These observations were reconfirmed by the discovery that Mih1, Ptp1, and $PP2A^{Rts1}$ act redundantly to regulate the spatial and temporal activation of Cdc28, collaborating to its stepwise activation prior to anaphase onset[51].

Swe1 and Mih1 are temporally and spatially modulated by various factors. The regulatory circuits involve Hsl1, Hsl7, Cla4 and Cdc5, which promote Swe1 phosphorylation at the septin ring[42,52-55]. Hyper-phosphorylated Swe1 is ubiquitinated by the Met30/SCF complex, which targets it to the Cdc34-dependent proteolysis[56].

Mih1, on the other hand, undergoes dramatic changes in

phosphorylation throughout most of the cell cycle in a Cdc28 and casein kinase 1-dependent manner[48]. Only during G2/M transition Mih1 is dephosphorylated and activated by Cdc55-dependent PP2A phosphatase[43,57].

Here we report that budding yeast haspin homologue Alk1 plays a critical role in the regulation of the G2/M transition in response to morphogenetic stress. Cells deleted for *ALK1* are indeed defective in the morphogenesis checkpoint and are characterized by a precoscious activation of Mih1 phosphatase, resulting in a premature Cdc28-Y19 dephosphorylation.

RESULTS

1) Deletion of *ALK1* causes a defect in the response to Latrunculin A induced morphogenetic stress.

Alk1 and Alk2 are two human haspin-like homologues present in budding yeast. We previously showed that both proteins are post-translationally modified during an unperturbed cell cycle when traversing mitosis and become hyper-phosphorylated in response to genotoxic stress[15]. However, the significance of this modification is still unclear. In fact, mutant strains carrying deletions of either one or both *ALK1* and *ALK2* haspin genes do not result in sensitivity to a variety of genotoxic agents. Moreover, Alk1 and Alk2 hyper-phosphorylation in response to genotoxic stress is not dependent on Mec1, Tel1 and Rad53, the major checkpoint protein kinases controlling the DNA damage response

in S.cerevisiae (data not shown).

We reasoned that the identification of the protein kinase responsible for Alk1 phosphorylation during an unperturbed cell cycle may provide insights on the other physiological roles of this haspin orthologue. We analyzed the phosphorylation state of Alk1 in genetic backgrounds carrying the deletion of twenty selected non-essential protein kinases controlling various aspects of the yeast cell cycle and metabolism. As shown in Figure 1A, we found by the use of λ phosphatase that Alk1 mobility to its phosphorylation is increased in a strain deleted for the *ELM1* gene. Elm1 is a protein kinase involved in many aspects of cellular morphogenesis, like septin behaviour or cytokinesis, and its function is required for proper mitotic hyper-phosphorylation of Swe1, the master regulator of the G2/M transition in response to morphological stress[58-61].
The finding that Alk1 phosphorylation is altered by *ELM1* deletion, led us to investigate a possible role of Alk1 in budding yeast cellular morphogenesis.

Latrunculin A (LatA) is a powerful natural toxin isolated from the Red Sea sponge *Negombata magnifica,* which was initially identified as a molecule that, by binding actin monomers, prevents their polymerization[62,63]. This LatA inhibitory effect leads, in budding yeast, to a defect in cellular morphogenesis and causes cell inability to emit the bud[64]. After incubation with this drug, wild-type cells activate a surveillance mechanism, known as

morphogenesis checkpoint, which leads to cell cycle arrest in the G2-phase of the cell cycle preventing nuclear division. On the other hand, cells deleted for *SWE1* or in genes required for morphogenesis checkpoint activation, enter mitosis even upon LatA treatment, dividing the nucleus within the mother cell without bud emergence completion[27-33, 64,65].

We thus analyzed the sensitivity of strains lacking either one or both *ALK1* and *ALK2* haspin homologues to LatA treatment. Wild-type, *alk1Δ, alk2Δ,* double mutant *alk1Δ alk2* and *swe1Δ* cells (as positive control) in *TUB1-GFP:HIS* background were arrested in G1 by α-factor treatment and then released into fresh medium containing 100 µM LatA. After 240 minutes, cells were collected, fixed and stained with DAPI to monitor nuclear division; spindle elongation was evaluated by fluorescence microscopy. As shown in Figure 1B, only ~ 10% of wild-type cells divide their nucleus within the mother cell, while ~ 70 % of *swe1*Δ cells exhibit two separated nuclei in the mother, indicating the failure into delaying nuclear division upon LatA treatment. Intriguingly, the strain deleted for *ALK1*, shows ~ 40% of cells with two nuclei in the mother, whereas deletion of the other haspin homologue, *ALK2,* behaves similarly to wild-type cells. Interestingly, deletion of *ALK2* suppresses the phenotype observed in an *alk1Δ* background, restoring a wild-type situation. Similar results were obtained when analysing anaphasic spindles elongation (Figure 1C).

Altogether these data indicate that *ALK1* plays a novel and

specific function in the response to morphogenetic stress. Such role of Alk1 is not shared with Alk2, which instead likely play an opposite function.

2) Deletion of *ALK1* inactivates the morphogenesis checkpoint triggered by misregulation of Cdc42.

To confirm a role for Alk1 in the response to morphogenesis alterations, we exploited a genetic approach to interfere with the actin cytoskeleton. Cdc24 is a guanine nucleotide exchange factor (GEF) for the GTPase Cdc42, the master regulator of polarity in budding yeast[66-68]. *CDC24* is an essential gene, but hypomorphic mutations cause defects in polarization processes and are tolerated thanks to the activation of the morphogenesis checkpoint[69]. We exploited a *cdc24-1* temperature sensitive allele to trigger checkpoint activation by shifting cells at non-permissive temperature, and checked the effect of haspin mutations.

Wild-type, *alk1Δ, alk2Δ,* double mutant *alk1Δ alk2Δ* and *swe1Δ* cells in *cdc24-1* background were grown at 25°C (permissive temperature); cultures were arrested in G1 with α-factor and shifted at 37°C (non-permissive temperature) for the last 45 minutes of the arrest, in order to deplete Cdc24 activity already before budding events. Cells were then released into fresh medium at 37°C, where they have to cope with polarized growth defects. Samples were taken 120 minutes after the release to evaluate nuclear division pattern. As it is shown in Figure 2A, in

absence of proper Cdc24 activity, wild-type cells delay mitosis through activation of the morphogenesis checkpoint, so that only ~ 10% of the cells go through nuclear division when budding is defective. On the other end of the spectrum we find *swe1Δ* mutants, where ~ 80% of the cells divide their nucleus, being unable to activate the checkpoint. Consistently with what observed with LatA, *cdc24-1 alk1Δ* cultures accumulate ~ 40% aberrant binucleated cells. This phenotype is again suppressed in cells lacking also *ALK2*.

Swe1 is a central kinase involved in activation of the morphogenesis checkpoint. Indeed, *swe1*Δ cells fail to inhibit mitotic entry upon morphogenetic stress and keep progressing also through the next cell cycle giving rise to tetranucleated cells with a 4C DNA content (Figure 2B). To investigate whether *ALK1* plays a similar regulatory role on the morphogenesis checkpoint, we examined the kinetics of cell cycle progression and nuclear division in *cdc24-1 alk1*Δ cells.

As shown in Figure 2, in the absence of Alk1, cells enter mitosis notwithstanding a morphogenetic insult, and become binucleated. These cells continue progressing through the cell cycle, indeed at 240 minutes after shift to non-permissive temperature they also become tetranucleated similarly to *swe1Δ* cells (Figure S1A and S1B). Moreover, we verified that deletion of *ALK1* or *ALK2* in a *swe1*Δ background does not alter the phenotype of *swe1*Δ cells (Figure S2A). These observations suggest that loss of *ALK1*

causes a defective, although not completely abolished, morphogenesis checkpoint, and that an epistatic relationship exists between *SWE1* and *ALK1.*

This model support the hypothesis that *alk1*Δ cells are temporarily delayed in mitotic entry, upon morphogenetic stress, but eventually they bypass the checkpoint arrest generating tetranucleted cells, although with a delay, compared to *swe1*Δ cells.

3) Cdc28-Y19 phosphorylation is reduced in *alk1Δ* cells.

In order to explain the defective cell cycle arrest, leading to unwanted nuclear division in *alk1*Δ cells experiencing a morphogenetic stress, we monitored the kinetics of nuclear division and Cdc28 phosphorylation on tyrosine 19 (Cdc28-Y19). This modification inactivates the CDK1, blocking entry into mitosis. Only upon dephosphorylation by the Mih1 phosphatase, will the cells proceed into mitosis. Cultures of cells carrying a *cdc24-1* mutation in the genetic background were synchronized in G1, shifted to non-permissive temperature to deplete Cdc24 activity before budding and released into the cell cycle. Wt, *alk1*Δ and *swe1*Δ cells were compared. Samples were taken every 15 minutes after the release, fixed and stained with DAPI to evaluate nuclear division. The results presented in Figure 3A show that, in the *cdc24-1* genetic background, cells initially stop cell cycle progression in the absence of *ALK1*, but later escape the arrest

and divide the nucleus, even though the bud is not present. Loss of *SWE1,* on the other hand completely prevents the G2/M arrest, abolishing the morphogenesis checkpoint. These observations demonstrate that in absence of Alk1, cells retain the ability to activate the checkpoint, but its active state could not be maintained.

The premature release from the morphogenetic stress-induced arrest observed in *alk1*Δ cells could indicate that Alk1 kinase may play an inhibitory function on Mih1 phosphatase, promoting maintenance of an active checkpoint. Intriguingly, Mih1 regulation has been reported to rely on an intricate balance of opposing kinases and phosphatases activities[39, 48,57,70-75].

To verify a possible role of Alk1 on Mih1 activity, we first tested the phosphorylation state of the major Mih1 target: Cdc28-Y19, the budding yeast CDK1. In the presence of a morphogenetic stress Swe1 phosphorylates Cdc28-Y19, avoiding mitotic entry. This happens since Swe1 protein is stabilized and Cdc28-Y19 phosphorylation accumulates, causing cell cycle arrest through the activation of the morphogenesis checkpoint. When all the conditions for proper nuclear division are satisfied, the CDK activity becomes essential for entry into mitosis. Swe1 must be degraded and the phosphatase activity of Mih1 has to revert Cdc28-Y19 phosphorylation to promote cell cycle restart. We hypothesized that, if Alk1 is acting as a Mih1 inhibitor, its absence should result in precocious dephosphorylation of Cdc28-Y19.

To test this hypothesis wild-type and *alk1Δ* strains in the *cdc24-1* background of experiment shown in Figure 3A were collected every 15 minutes also for protein extracts preparation (Materials and Methods). Protein samples were separated by SDS-PAGE and analyzed by Western blot with an antibody specifically recognizing Cdc28-Y19 phosphorylation (Figure 3B). Cell cycle analysis of cells of Figure 3 was performed by FACScan cytometer and is shown in Figure S3A. We quantified the level of Cdc28-Y19 phosphorylation respect to the total amount of Cdc28 at the different time-points, and normalized these values for the amount present at time 0; this ratio is shown in a chart representative for three independent experiments (Figure 3C). In wild-type cells carrying the *cdc24-1* allele the level of Cdc28-Y19 phosphorylation increases with time, reaching a peak when cell cycle progression is arrested. In *cdc24-1 alk1Δ* cells the level of Cdc28-Y19 phosphorylation instead increases with a similar kinetic respect to wild-type cells, but remains stable at a lower amount (Figure 3C).

This finding supports the hypothesis that in absence of *ALK1* Cdc28-Y19 phosphorylation is impaired and, as a consequence, entry into mitosis is induced even in the presence of a morphogenetic stress. These data could be explained by an higher rate of Mih1-dependent dephosphoorylation of Cdc28-Y19 as previously proposed, but they are also consistent with a role of Alk1 in regulating Swe1 kinase.

In an attempt to verify whether Alk1 acts on either of the two regulators of morphogenesis checkpoint, we tested the phosphorylation state of Swe1 and Mih1 upon deletion of *ALK1*. Both proteins are controlled by a complex network of different phosphorylation events, which regulates their stability and function[43, 70-75]. Such complexity made it hard to obtain conclusive data and we report that Swe1 or Mih1 electrophoretic mobility and levels are apparently not affected by *ALK1* deletion during an unperturbed cell cycle (Figure S4A), or following activation of the morphogenesis checkpoint in a *cdc24-1* background (Figure S4B).

4) Deletion of *MIH1* rescues the *alk1Δ* cells defective response to morphological stresses.

In order to discriminate if reduction in Cdc28-Y19 phosphorylation in *alk1*Δ cells is due to the misregulation of Swe1 or Mih1 activity, we combined *ALK1* deletion with *mih1*Δ. If loss of Alk1 enhanced Mih1 activity, removal of *MIH1* should restore wild-type Cdc28-Y19 phosphorylation in *alk1*Δ cells.

To verify this, wild-type, *alk1*Δ*, mih1*Δ and *alk1*Δ*mih1*Δ in a *cdc24-1* background were arrested in G1 with α-factor at 25°C (permissive temperature) and shifted at 37°C (non-permissive temperature) for the last 45 minutes of the arrest. At the end of the G1 arrest, cells were released into fresh medium at 37°C.

Samples were collected every 15 minutes for 150 minutes from the release. Both nuclear division pattern and protein extracts were analyzed. Protein samples were separated by SDS-PAGE and analyzed by Western blot with an antibody specifically recognizing Cdc28-Y19 phosphorylation. The level of Cdc28-Y19 phosphorylation was compared to total Cdc28 at the different time-points and the resulting ratio is shown in Figure 4A as a chart representative of three independent experiments. The same analysis was repeated for the last point of the kinetics (Figure 4B). *cdc24-1 mih1Δ* cells show a Cdc28-Y19 phosphorylation similar to wild-type, whereas *cdc24-1 alk1Δ* cells exhibit a decreased signal, confirming previous data (Figure 4A and 4B).

Interestingly, while *MIH1* deletion does not impact on Cdc28-Y19 modification, additional removal of *ALK1* partially recovers Cdc28-Y19 phosphorylation, suggesting that loss of *ALK1* causes a precoscious activation of Mih1 phosphatase. This hypothesis is reconfirmed by the observation that also defective nuclear division is rescued by additional deletion of *MIH1* in *alk1Δ* cells (Figure 4C). Cell cycle analysis of cells of Figure 4 was performed by FACScan cytometer and is shown in Figure S5A.

5) *SWE1* overexpression defects are decreased by deletion of *ALK1*.

It is known that Swe1 overexpression leads to morphogenesis checkpoint hyper-activation. This effect is coupled with cell cycle

arrest at G2/M transition, with 2C content of DNA and elongated spindles. In this situation cells arrest prior to mitotic entry with very high Cdc28-Y19 phosphorylation, and are characterized by a hyper-polarized growth of buds[27].

To genetically reconfirm that Alk1 inhibits Mih1 phosphatase, we created a plasmid carrying *SWE1* gene tagged with *GFP* under *GAL1-10* inducible promoter and tried to verify whether deletion of *ALK1* would interfere with Swe1 overexpression phenotypes (Material&Methods). Wild-type and *alk1Δ* cells transformed with this plasmid were arrested in G1 by α-factor in presence of raffinose as unique carbon source. At the end of the arrest cells were released in galactose containing media (2% final concentration) added with nocodazole (10 μg/ml) and samples were collected every 15 minutes for 150 minutes. Samples were fixed to evaluate protein extracts, and also cellular morphology was analyzed by microscopy. Protein extracts were separated by SDS-PAGE and visualized by Western blot with the use of specific antibodies.

Consistently with the hypothesis that Alk1 inhibits Mih1 activation, Cdc28-Y19 phosphorylation is visibly descreased in *alk1Δ* cells respect to wild-type cells even though Swe1 kinase is overexpressed (Figure 5A). The level of Cdc28-Y19 phosphorylation was compared to total Cdc28 at the different time-points and the resulting ratio is shown in Figure 5B in a chart representative for three independent experiments. This chart

clearly indicates that high levels of Cdc28-Y19 phosphorylation are prematurely removed in *alk1Δ* cells, although if Swe1 kinase is over-expressed. Moreover, removal of *ALK1* also partially suppresses the morphological defects due to *SWE1* overexpression, reconfirming previous observations and hypothesis (Figure 5C). Cell cycle analysis of cells of Figure 5 was performed by FACScan cytometer and is shown in Figure 5D and 5E.

DISCUSSION

Altogether, the data presented indicate that haspin homologues play a role in the response to morphogenetic stress of budding yeast. We showed that *ALK1* plays an important positive role in the morphogenesis checkpoint, while *ALK2* may have an opposing function; indeed *ALK2* deletion partially rescues the *cdc24-1 alk1*Δ defective phenotype (Figure 1 and Figure 2). Moreover, we found that in absence of *ALK1* cells continue progressing through the cell cycle, becoming tetranucleated similarly to *swe1Δ* cells at later time points after shift to non-permissive temperature (Figure S1).

We verified also that Alk1 and Swe1 work in the same pathway (Figure S2). Unfortunately, our data show also that *ALK2* deletion does not recover the nuclear division defect of *swe1*Δ cells (Figure S2), leaving questions to answer about the interplay between Alk1 and Alk2 in the G2/M transition regulation. However

since Alk2 seems to plays a role that is opposite to that of Alk1, we can hypothesize that Alk2 negatively affects Swe1 function. This would explain why deletion of *ALK2* does not affect a *swe1*Δ strain.

Intriguingly, this might be the first time where Alk1 and Alk2 paralogues do not share a common function. In particular, Alk1 seems to inhibit Mih1 unscheduled activation during the morphogenesis checkpoint. In absence of Alk1, indeed, we showed that the inhibitory Cdc28-Y19 phosphorylation decreases prematurely, leading to precoscious checkpoint inactivation and defective nuclear division (Figure 3 and S3). These defects are recovered by the additive deletion of *ALK2*, suggesting that the protein can compete with Alk1 for the binding to a still unknown key substrate or eventually regulate the other branch of the pathway, as already suggested, by acting on Swe1.

From our data it is difficult to conclusively understand if haspin influences the status of Swe1 or Mih1, the two main regulators of the checkpoint (Figure S4). However, our observations clearly indicate that *ALK1* deletion reduces the inhibitory phosphorylation on CDK1 in yeast cells during the morphogenesis checkpoint activation (Figure 3), pointing toward an involvement of haspin in the balance between Swe1 and Mih1 activities.

In order to clarify Alk1 role in the morphogenesis checkpoint we removed the contribution of Mih1 to Cdc28-Y19 phosphorylation

in *cdc24-1 alk1*Δ cells, demonstrating that loss of *ALK1* leads to a precoscious Mih1 activation (Figure 4 and S5). This hypothesis was also reconfirmed by the observation that deletion of *ALK1* partially reverts the phenotypes observed in cells overexpressing Swe1 (Figure 5). These cells are supposed to accumulate an hyper-phosphorylated Cdc28-Y19 and to remain stacked at the G2/M transition, continuously enlarging their bud size without going through mitosis. The fact that *ALK1* loss partially rescues their defects is consistent again with an unscheduled Mih1 activation and Cdc28-Y19 phosphorylation premature decrease.

Our studies indicate that the investigation on Swe1/Mih1 pathway is still in progress. We think that the clarification of this pathway in human cell lines could be extremely interesting, especially looking at the possible involvement of haspin kinase in Cdc25 and Wee1 regulation. Indeed, despite this pathway has different roles in *S. cerevisiae* respect to human cells, the molecular mechanisms underlying modulation of CDK1 activity is conserved from yeast to mammals. Wee1 and Cdc25 have crucial activities in the control of cell cycle and their misfunction is often coupled with cancerogenesis. Unfortunately, the mechanism by which *WEE1* and *CDC25* become deregulated during cancer development remains still unclear. Conceptually, we therefore strongly believe that studies on haspin activity in the Wee1/Cdc25 pathway can shed light in long term on mechanisms underlying cancerogenesis.

METHODS

Yeast Strains and Plasmids

Yeast strains used in this study are isogenic to W303, and are listed in Table S1. Conditions for yeast cell cultures used have been previously described[76]. When indicated the cultures were synchronized by α-factor treatment (2μg/ml) as previously described[77]. Moreover nocodazole treatment (10μg/ml) was used only in few experiments to induce an arrest in G2-phase of the cell cycle for 150 minutes. Standard molecular genetics techniques were used to construct plasmid and strains. In particular, PCR-based genotyping were used to confirm gene disruption and tagging[78]. Strains containing *cdc24-1* allele were derived from a strain in DLY5 background kindly provided by Dr. D. J. Lew (Duke University Medical Center, Durham, NC 27710, Department of Pharmacology and Cancer Biology) and backcrossed five times into the W303 background. W303 strains containing *TUB1-GFP::HIS* were derived from the SP1791 strain, kindly provided by Dr. S. Piatti (Centre de Recherche en Biochimie Macromoléculaire, Montpellier, France). Overexpression of *GFP-SWE1 was* obtained by transforming cells with the pPD22 centromeric plasmid. This plasmid carries *SWE1* gene cloned under the *GAL1* promoter in the pGREG575 backbone (#P30373, Euroscarf), created following published procedures[79]. Gene overexpression with the inducible *GAL1* promoter were achieved by adding 2% galactose to raffinose-containing medium. Temperature-sensitive mutants were grown either at permissive

(25°C) or restrictive (37°C) temperature.

Strains used in this work

Name	Relevant Genotype	Source
YAN165/7B	*K699, ALK1-3HA::TRP matA*	lab stock
YPD374/9D	*K699, ALK1-3HA::TRP1, elm1::NATr matA*	this work
SP1791	*K699, Tub1-GFP::HIS3 matA*	Piatti's Lab
YPD294	*K699, Tub1-GFP::HIS3, alk1::NATr matA*	this work
YPD414/1A	*K699, Tub1-GFP:HIS3, alk2::KANr matA*	this work
YPD298	*K699, Tub1-GFP::HIS3, alk1::NATr, alk2::KANr matA*	this work
YPD300	*K699, Tub1-GFP::HIS3, swe1::LEU2 matA*	this work
YPD274	*K699, cdc24-1 matA*	this work
YPD280/9A	*K699, cdc24-1, alk1::NATr matA*	this work
YPD282/12A	*K699, cdc24-1, alk2::KANr matA*	this work
YPD282/5A	*K699, cdc24-1, alk1::NATr, alk2::KANr matA*	this work
YPD458	*K699, cdc24-1, swe1::LEU2 matA*	this work
YPD459	*K699, cdc24-1, swe1::LEU2, alk1::NATr matA*	this work
YPD460	*K699, cdc24-1, swe1::LEU2, alk2::KANr matA*	this work
YPD290	*W303 (diploide), cdc24-1, alk1::NATr, alk2::KANr, swe1::LEU2*	this work
YPD291	*W303 (diploide), cdc24-1, alk1::NATr, alk2::KANr, mih1::TRP1*	this work
YPD286/10C	*K699, cdc24-1, mih1::TRP1 matA*	this work

YPD288/7A	*K699, cdc24-1, alk1::NATr, mih1::TRP1 matA*	this work
YAN146/2D	*K699, SWE1-3HA::URA3 matA*	lab stock
YPG37/1B	*K699, SWE1-3HA::URA3, alk1::KANr matA*	lab stock
YAN111/1A	*K699, MIH1-3HA::TRP1 matA*	lab stock
YPG36/B8	*K699, MIH1-3HA::TRP1, alk1::KANr matA*	lab stock
YPD336/6A	*K699, cdc24-1, SWE1-3HA::URA3 matA*	this work
YPD338/11A	*K699, cdc24-1, SWE1-3HA::URA3, alk1::KANr matA*	this work
YLD18/20C	*K699, cdc24-1, MIH1-3HA::TRP1 matA*	this work
YLD19/13A	*K699, cdc24-1, MIH1-3HA::TRP1, alk1::KANr matA*	this work
YPD400	*K699, [pPD22::LEU2] matA*	this work
YPD401	*K699, alk1::KANr, [pPD22::LEU2] matA*	this work

Plasmids used in this work

Name	Relevant Genotype	Source
pPD22	*pGREG-GAL-GFP-SWE1::LEU*	this work

Protein extracts treated with λ phosphatase

To analyze proteins during λ protein phosphatase experiments samples were collected from exponentially growing cells and exposed to trichloroacetic acid precipitation[80]. After precipitation pellets were resuspended in 50 µl of λ phosphatase buffer (NEBuffer for PMP 1X) supplemented with 1 mM $MnCl_2$. The pH

of extracts was then buffered with Trizma-Base until a value of 7-8. Samples were added with 5 µl of λ protein phosphatase (2000U of NEB lambda PP) and incubated at 30°C for 30 minutes. Lastly, 10 µl of Laemmli buffer (6X) were added before samples boiling and clarification by centrifugation. Protein extracts were then resolved by SDS-PAGE and analyzed by Western blot using proper antibodies.

Latrunculin A Treatment

LatA (SIGMA L5163) was stored at 20 mM DMSO stock solution at −20°C. Cells were grown in YPD medium, synchronized in G1 with α-factor (2µg/ml) and released in the presence of LatA 100 µM for 240 minutes. Cells were then harvested for trichloroacetic acid protein extraction or fixed for microscopy analysis.

Spindle elongation and nuclear division analysis

To evaluate spindle elongation cells carrying *TUB1-GFP::HIS* allele were fixed with formaldehyde (3.7%), and washed three times with PBS. GFP was visualized by fluorescence microscopy with a Leica DMRA2 widefield fluorescence microscope equipped with a CCD camera (Leica DC 300F). For the analysis of nuclear division cells were fixed with ethanol, washed three times in PBS and subjected to DNA staining with DAPI. Labeled-DNA was visualized by fluorescence microscopy as described above. Images were processed by Image J. Nuclear division pattern was evaluated by scoring for unbudded cells showing a single nucleus

or two nuclei. At least 300 cells were categorized per sample across three experimental repeats to calculate a mean and a standard deviation.

Morphogenesis checkpoint assays

To evaluate morphogenesis checkpoint activation cells carrying *cdc24-1* temperature-sensitive allele were grown at 25°C (permissive temperature), arrested in G1 with α-factor (2μg/ml), shifted for 45 minutes at 37°C (non-permissive temperature) and released at 37°C. At indicated time points samples were collected, fixed in ethanol and stained with DAPI. Nuclear division was evaluated as described above. Moreover, trichloroacetic acid protein extraction was used to evaluate Cdc28-Y19 phosphorylation by Western blot. The ratio between Cdc28-Y19 phosphorylation and total Cdc28 was performed on protein levels of three independent experiments.

Western blot

To analyze proteins during kinetic experiments samples were collected at given time points and exposed to trichloroacetic acid precipitation[80]. Protein extracts were then resolved by SDS-PAGE and analyzed by Western blot using proper antibodies. Anti-HA antibodies (12CA5) were used as previously described[81]. Anti-phospho-cdc2 (Tyr15) (#9111, Cell Signaling), anti-Cdc2 (ab17) (#ab18-100, Abcam), anti-GFP (#A-6455, Termofisher), anti-tubulin (#ab6160, Abcam), anti-actin (#A2066, SIGMA-Aldrich) and anti-GST (#27-4577-01V, GE Healthcare) were used

with standard techniques. Images were taken with a ChemidocTouch Imaging System (Bio-Rad) and processed with ImageLab and ImageJ.

Data and Statistical analysis

Cdc28-Y19 phosphorylation was always quantified respect to total Cdc28 amount to produce a chart representative for three independent experiments. In some experiments statistical analyses were performed using the statistical independent t-test to confirm that the differences measured between wild-type cells and other strains were significative. There results are presented as means ± standard deviation. Differences were considered statistically significant whenever p-value was < 0.05.

Cellular morphology evaluation

Cells carrying the pPD22 centromeric plasmid were grown in raffinose- containing medium and arrested in G1 with α-factor (2μg/ml). During the G1 arrest *SWE1* overexpression was induced for the last 45 minutes by galactose add (2%). Cells were then released in galactose-containing medium added with nocodazole (10μg/ml), harvested at indicated time points, fixed with formaldehyde (3.7%), and washed three times with PBS. Upon *SWE1* overexpression cells accumulate a long buds peculiar phenotype[27]. The evaluation of cellular morphology was performed by scoring for cells showing normal or abnormal bud size. At least 300 cells were categorized per sample across three

experimental repeats to calculate a mean and a standard deviation.

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 1μM SytoxGreen and DNA content was determined using a FACScan cytofluorimeter.

ACKNOWLEDGMENTS

D. Lew and S. Piatti are acknowledged for donating strains. M.M.F lab is supported by grants from AIRC (n.15631) and Telethon (GGP15227). We sincerely thank Marco Geymonat and Simonetta Piatti for useful discussion.

AUTHOR CONTRIBUTIONS

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

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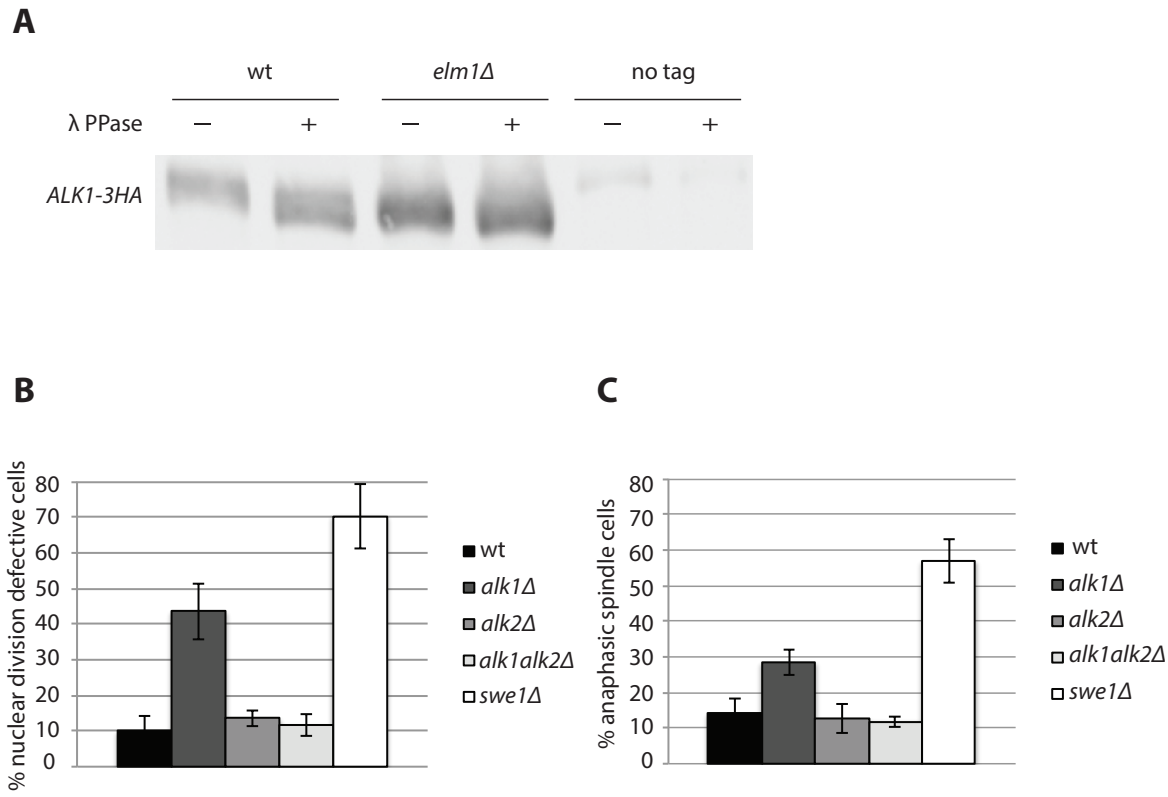

Figure 1 Deletion of *ALK1* causes a defect in the response to Latrunculin A induced morphogenetic stress. **a** wt and *elm1Δ* cells were grown in untreated conditions. Trichloroacetic acid protein extracts were treated with λ PPase and separated by SDS-PAGE. *ALK1-3HA* was monitored by Western blot using specific antibodies. **b** wt, *alk1Δ*, *alk2Δ*, *alk1Δ alk2Δ* or *swe1Δ* cells were arrested in G1 by α-factor (2μg/ml) and released into LatA (100 μM) containing medium; after 240 min cells were collected and fixed. Cells were stained with DAPI to monitor nuclear division. **c** Tubulin from samples in panel **b** was visualized by fluorescence microscopy to evaluate spindle elongation; error bars in panel **b** and **c** represent standard deviation.

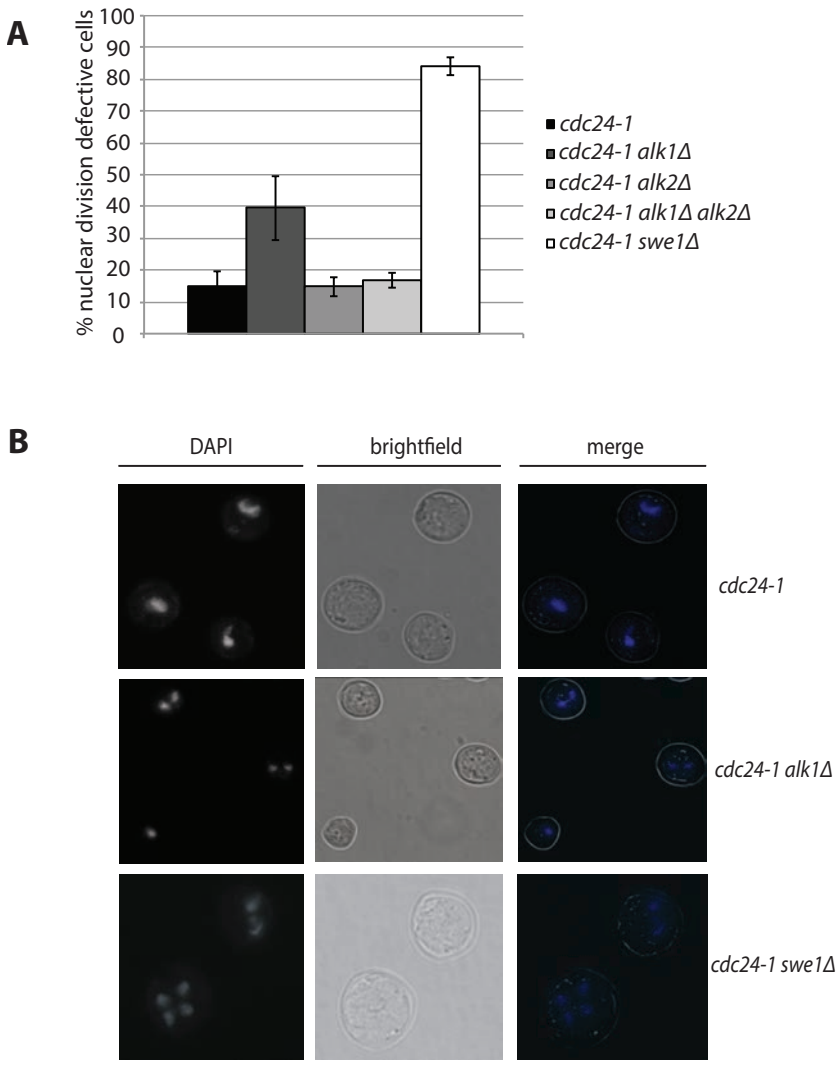

Figure 2 Deletion of *ALK1* inactivates the morphogenesis checkpoint triggered by misregulation of Cdc42. **a** wt, *alk1Δ*, *alk2Δ*, *alk1Δ alk2Δ* or *swe1Δ* cells all in *cdc24-1* background were arrested in G1 by α-factor (2µg/ml) at 25°C (permissive temperature), shifted for 45 min at 37°C (non-permissive temperature) and released at 37°C. Samples were collected after 120 min and stained with DAPI to monitor nuclear division pattern. **b** Fluorescence microscopy imaging of *cdc24-1*, *cdc24-1 alk1Δ* and *cdc24-1 swe1Δ* cells from panel **a**.

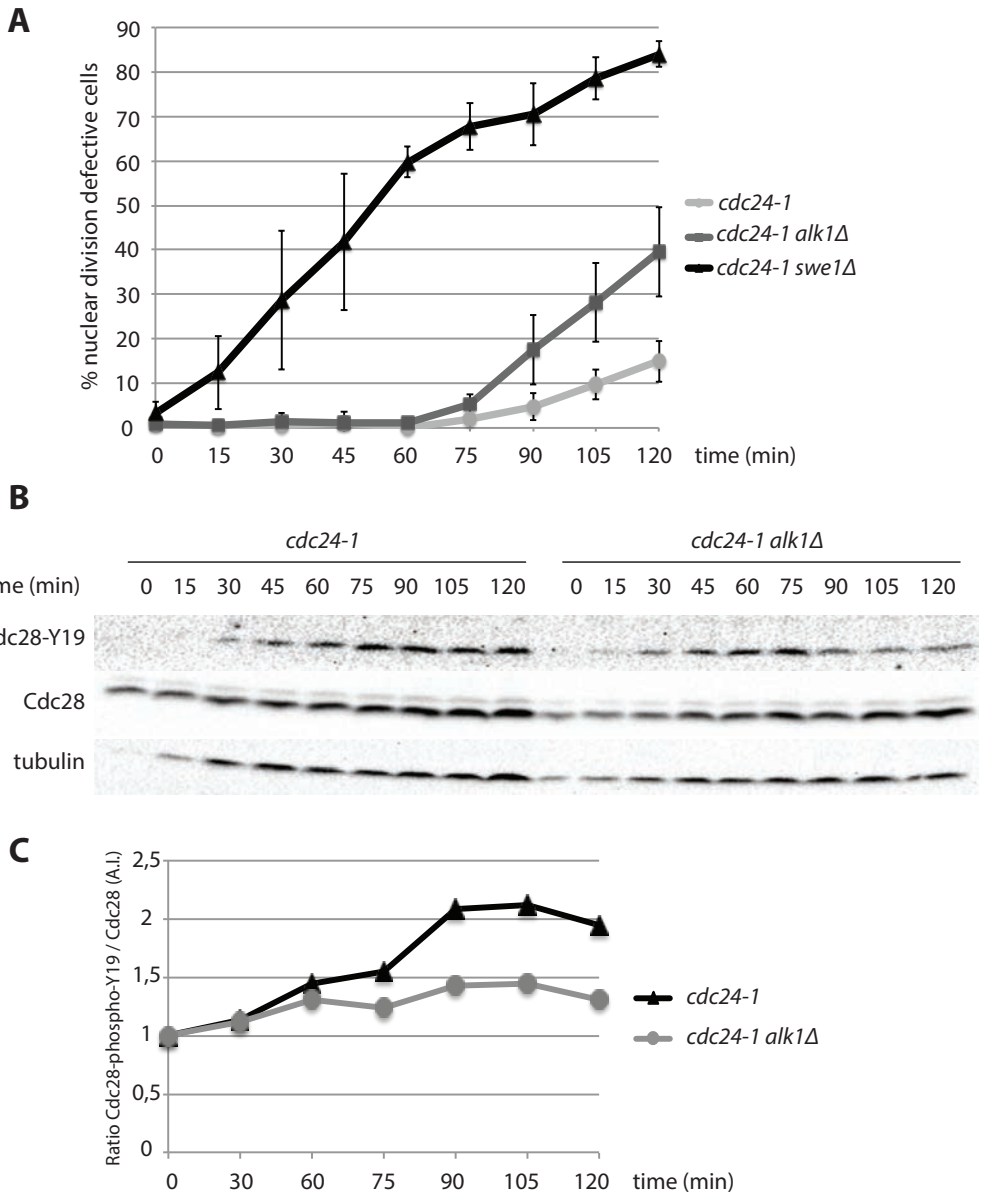

Figure 3 Cdc28-Y19 phosphorylation is reduced in *alk1Δ* cells.
a wt, *alk1Δ*, and *swe1Δ* cells all in *cdc24-1* background were arrested in G1 by α-factor (2μg/ml) at 25°C, shifted for 45 min at 37°C and released into fresh medium at 37°C. Samples were collected every 15 min and stained with DAPI to evaluate nuclear division pattern. Error bars show standard deviation. **b** For wt and *alk1Δ* cells from panel **a** trichloroacetic acid protein extracts were prepared and separated by SDS-PAGE. Cdc28-Y19 phosphorylation was monitored by Western blot using specific antibodies. **c** Ratio of phosphorylated Cdc28-Y19 from panel **b** respect to total Cdc28 was performed, and normalized respect to time zero. The chart is representative of three independent experiments. A.I. indicates arbitrary units.

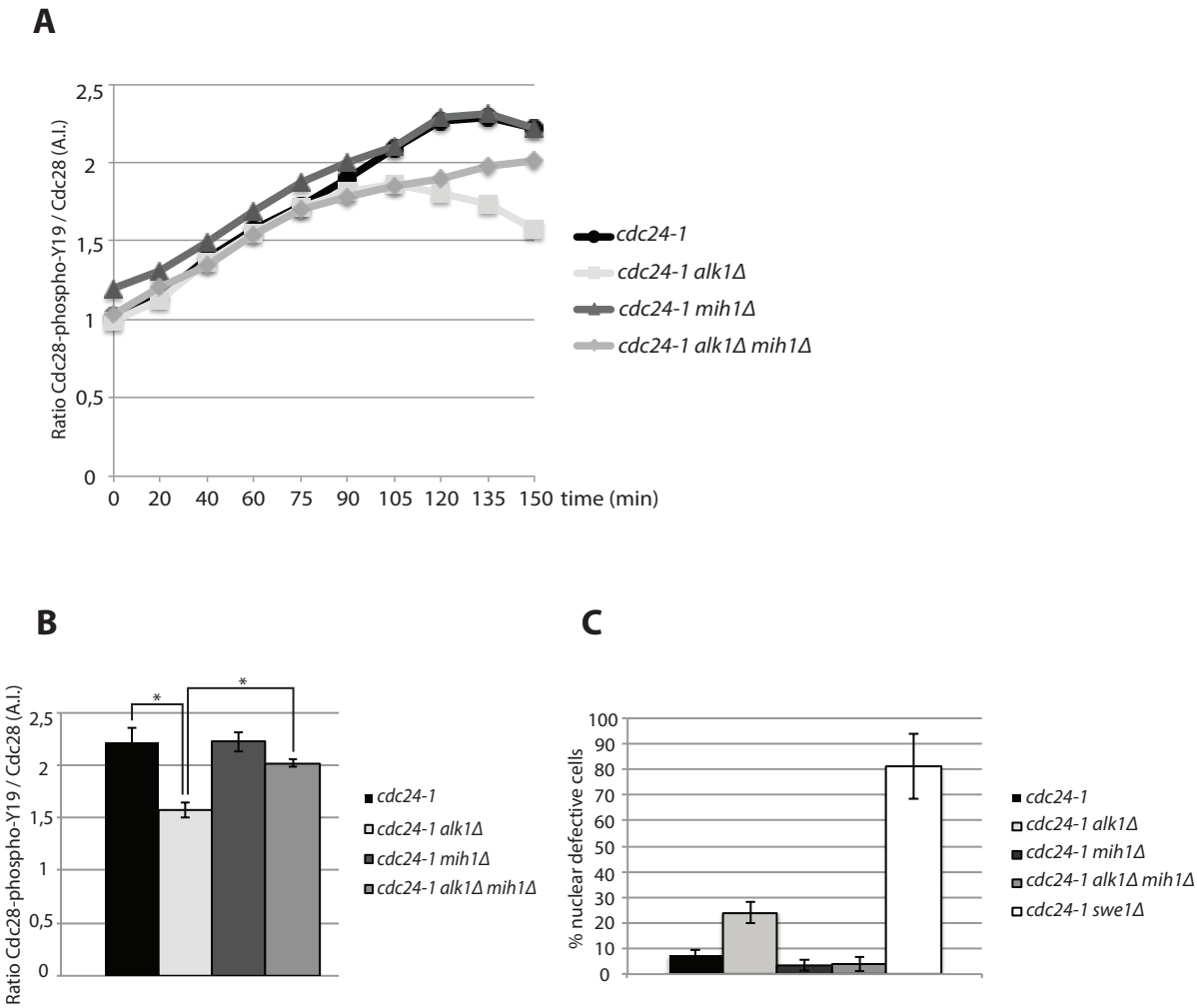

Figure 4 Deletion of *MIH1* rescues the *alk1Δ* cells defective response to morphological stress.
a wt, *alk1Δ*, *mih1Δ* and *mih1Δ alk1Δ* cells all in *cdc24-1* background were arrested in G1 by α-factor (2μg/ml) at 25°C, shifted for 45 min at 37°C and released into fresh medium at 37°C. Samplex were collected at the indicated time points and trichloroacetic acid protein extracts were prepared and separated by SDS-PAGE. Cdc28-Y19 phosphorylation was monitored by Western blot using specific antibodies and quantified respect to total Cdc28. The chart is representative of three independent experiments. A.I. indicates arbitrary units. **b** Quantification shown in panel **a** was repeated only for the last time point (150 min). Each value respresents the mean +/- standard deviation of duplicated independent experiments (* $p < 0,05$). **c** For the strains of panel **a** and the positive control *swe1Δ* were collected also samples at 150 min to evaluate nuclear division pattern upon DAPI staining.

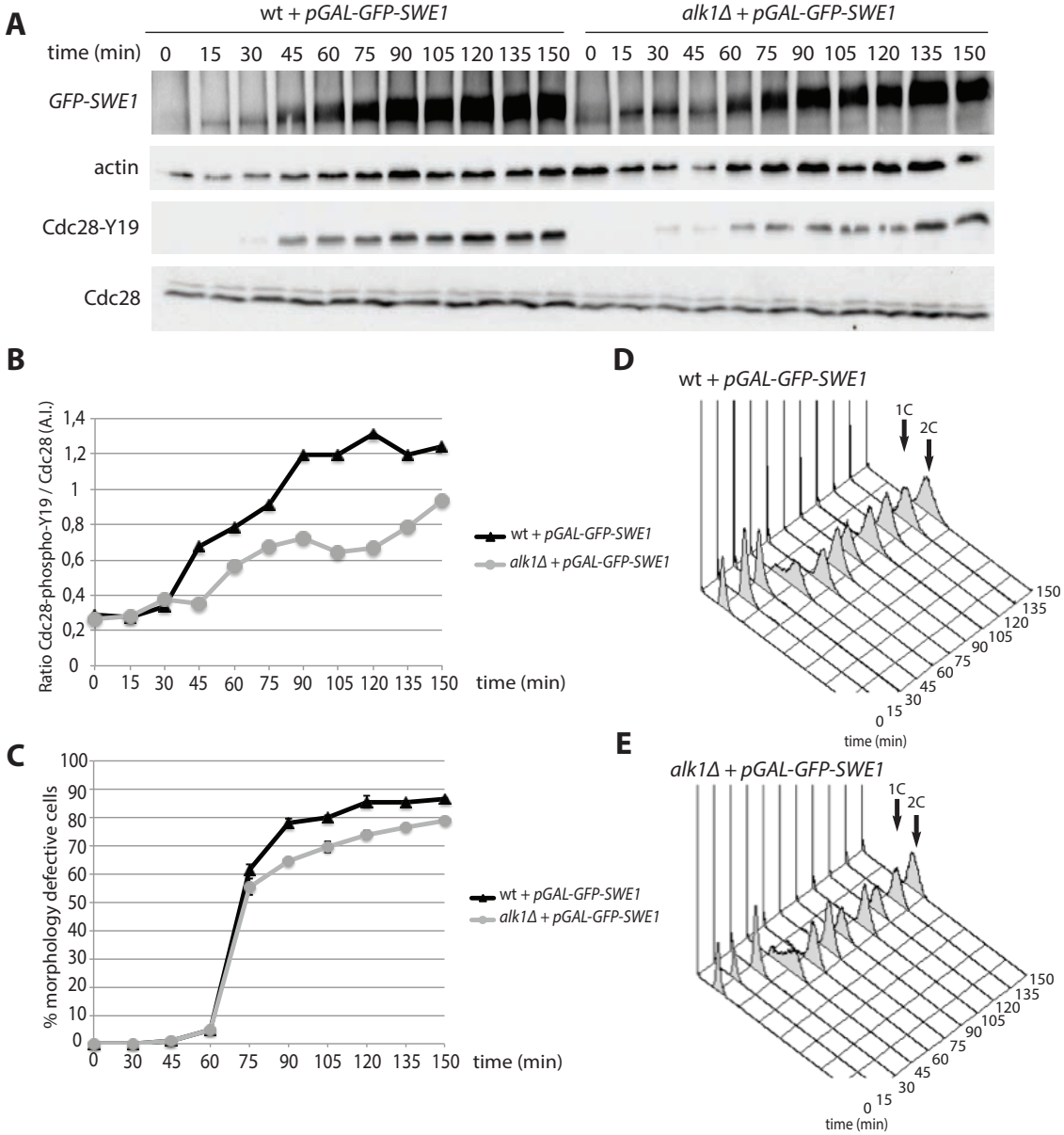

Figure 5 *SWE1* overexpression defects are decreased by deletion of *ALK1*.
a wt and *alk1Δ* cells carrying the *pGAL-GFP-SWE1* plasmid for *SWE1* overexpression were grown in raffinose and arrested in G1 by α-factor (2μg/ml). During the arrest *SWE1* overexpression was induced for the last 45 min by galactose (2%) add. Cells were then released into nocodazole-containing medium (10 μg/ml). Samples were taken at the indicated time points and fixed to evaluate cellular morphology and protein extracts, which were separated by SDS-PAGE. *SWE1* overexpression level and Cdc28-Y19 phosphorylation were monitored by Western blot using specific antibodies. **b** Quantification of phosphorylated Cdc28-Y19 from panel **a** was performed respect to total Cdc28. The chart is representative of three independent experiments. A.I. indicates arbitrary units. **c** Cellular morphology defects of strains from panel **a** was evaluated in three independent experiments. Error bars indicate standard deviation. **d,e** Cell cycle analysis by FACS of strains in panel **a**.

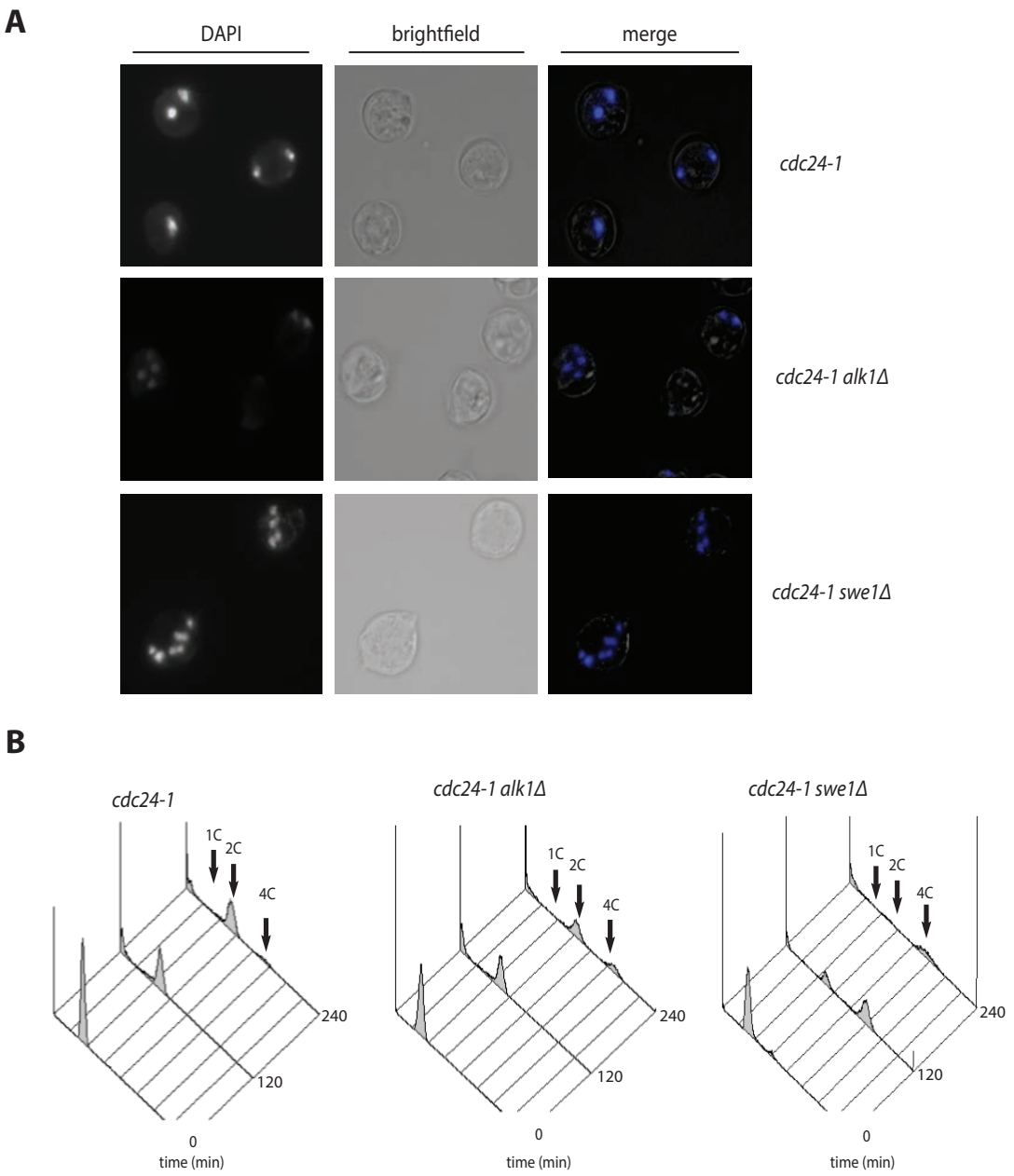

Figure S1 Analysis of cells deleted for *ALK1* at later time points from the morphogenetic stress induction. **a** Fluorescence microscopy imaging of experiment in Figure 2 at 240 min from the release. **b** Cell cycle analysis by FACScan of cells from experiment in Figure 2 at 120 min and Figure S1 at 240 min.

A

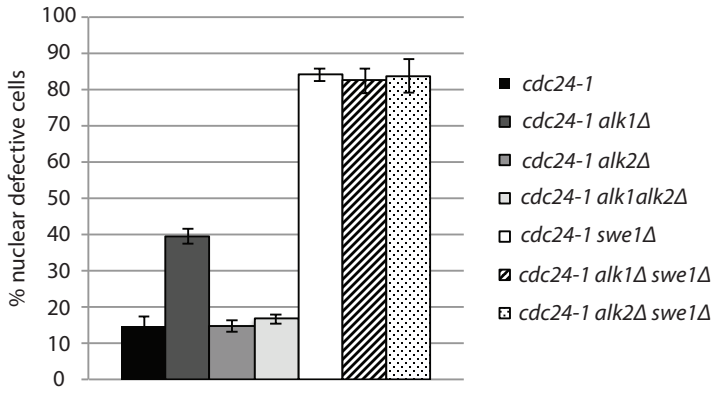

Figure S2 Genetic interactions between *ALK1, ALK2* and *SWE1*.
a wt, *alk1Δ*, *alk2Δ*, *alk1Δ alk2Δ*, *swe1Δ, swe1Δ alk1Δ, swe1Δ alk2Δ* cells all in *cdc24-1* background were arrested in G1 by α-factor (2μg/ml) at 25°C, shifted for 45 min at 37°C and released into fresh medium. Samplex were collected after 120 min , fixed and stained with DAPI to evaluate nuclear division pattern.

A

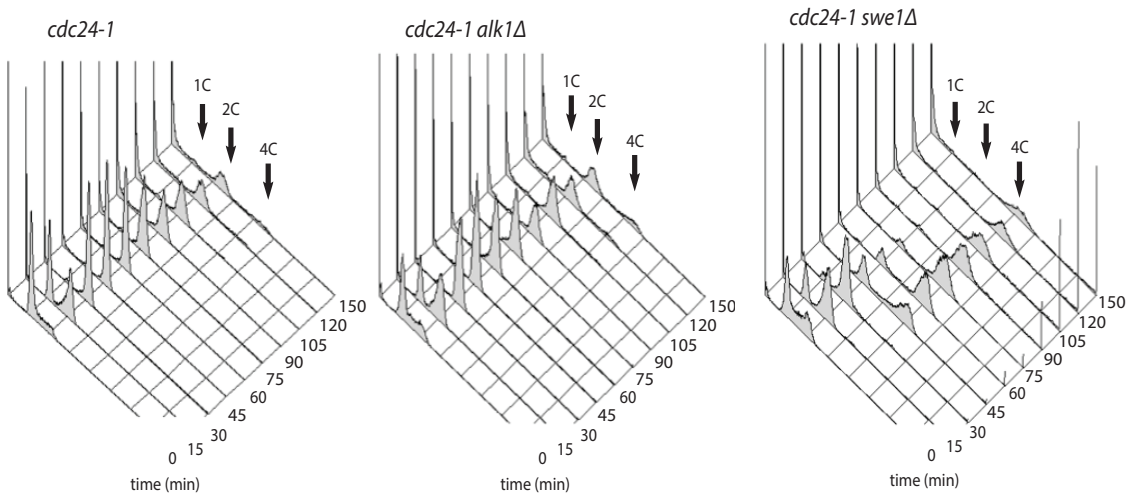

Figure S3 a Cell cycle analysis by FACS of cells from experiment in Figure 3.

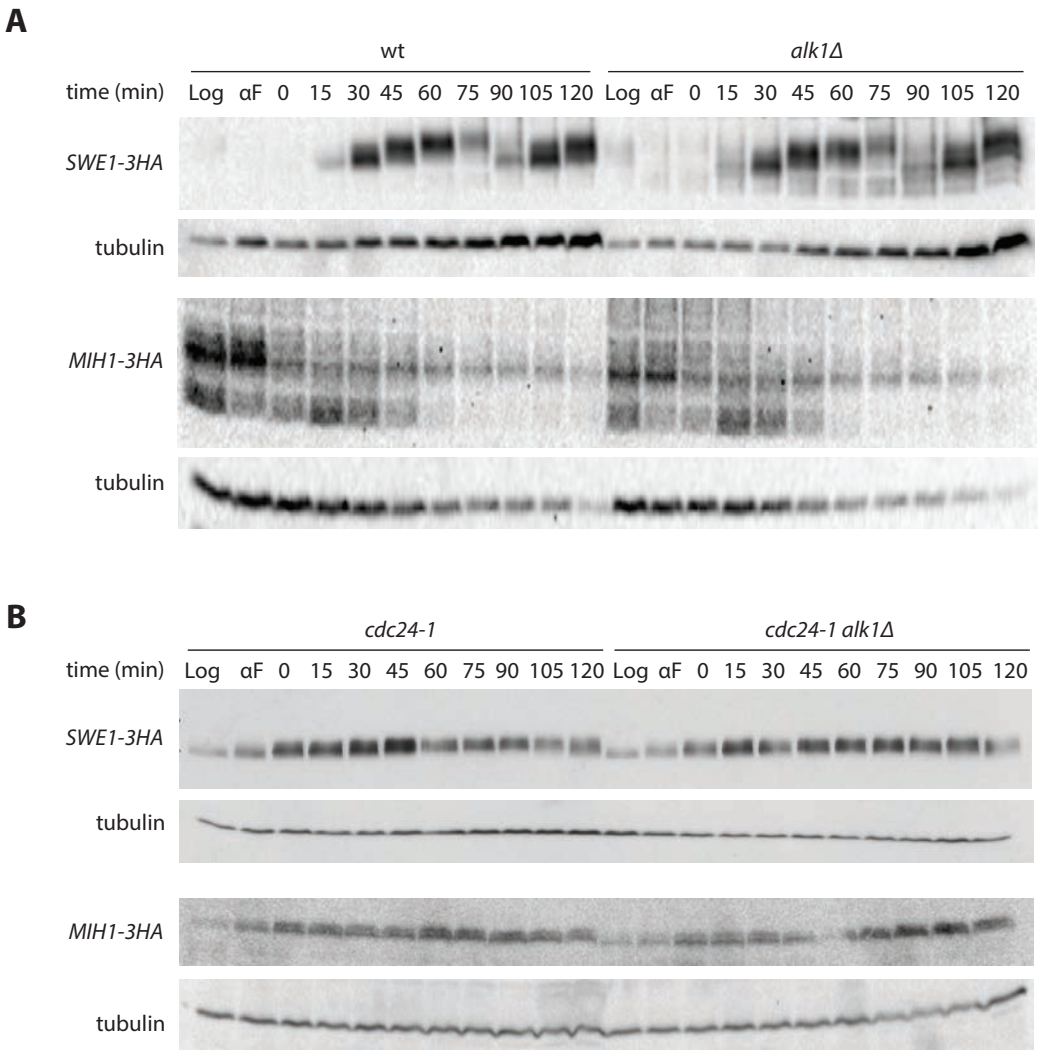

Figure S4 Analysis of the phosphorylation state of Swe1 and Mih1, in the absence (**a**) or in the presence (**b**) of a morphogenetic stress.
a wt and *alk1Δ* cells were arrested in G1 by α-factor (2 µg/ml) and then released into fresh medium. Samples for total protein extracts were collected every 15 min and prepared, separated by SDS-PAGE and analysed by Western blot with anti-HA antibody. **b** *cdc24-1* and *cdc24-1 alk1Δ* cells were arrested in G1 by α-factor (2 µg/ml) at 25°C (permissive temperature), shifted for 45 min at 37°C (non-permissive temperature) and released into fresh medium at 37°C; cells were collected every 15 min and total protein extracts analyzed as described above.

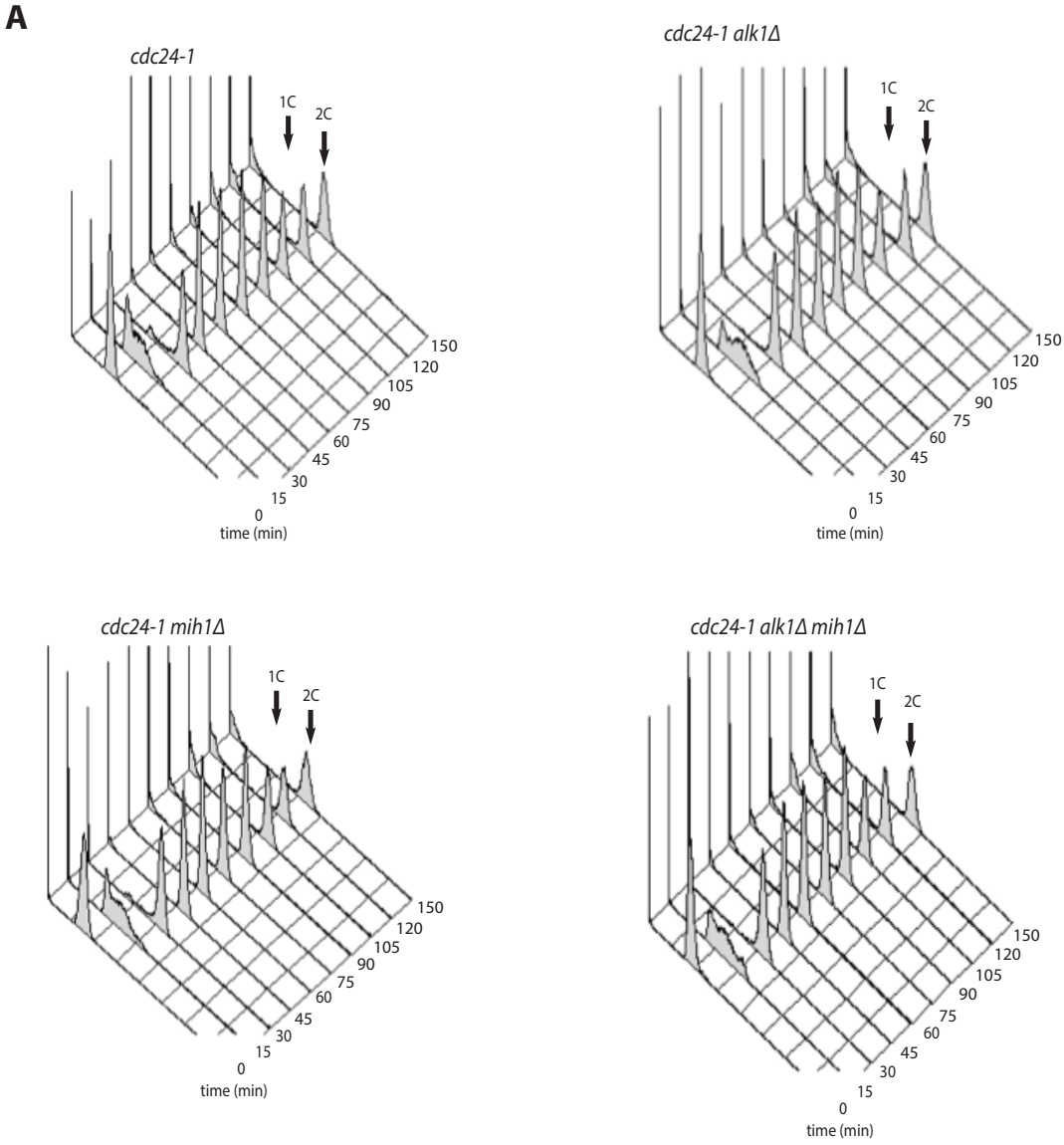

Figure S5 a Cell cycle analysis by FACS of cells from experiment in Figure 4.

Part III

Manuscript II

Content: Submitted Manuscript

Roberto Quadri, Martina Galli, Elena Galati, Giuseppe Rotondo, Guido Roberto Gallo, Davide Panigada, Paolo Plevani, Marco Muzi-Falconi

"Haspin regulates Ras localization to promote mitotic Cdc24-driven depolarization"

HASPIN REGULATES RAS LOCALIZATION TO PROMOTE MITOTIC CDC24-DRIVEN DEPOLARIZATION

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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 exhaustively known. In *Saccharomyces cerevisiae* haspin is important for the regulation of mitotic spindle positioning and in the tolerance of mitotic delays. Here we identify haspin kinase as a factor critical 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 of Ras. Haspin promotes a shift from a bud-tip oriented to an even delivery of vesicles to the PM during mitosis

that is required for proper distribution of Ras. We report a mitotic role for Ras and show that, controlling redistribution of Cdc24, it regulates activation of the Cdc42 GTPase at polarized sites. 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: its deregulation can lead to severe diseases and is one of the first steps of malignant transformation in carcinogenesis[1]. Indeed, during tumorigenesis, cells change their behaviour through the epithelial to mesenchymal transition (EMT) that provides them the capability to outnumber the surrounding tissues, to eliminate the need for external signals and to move and invade distal compartments of the organism[2]. One of the first hallmarks of EMT is the loss, or alteration, of cellular polarization, with rearrangements of some key factors (e.g. PAR proteins). This leads to turn-off the established apico-basal polarity favouring a front-rear one, degradation of proteins involved in cell-cell contacts (e.g. E-cadherin) and overall promotion of cellular motility[1].

A family of small proteins, Rho GTPases, oversees cellular polarity, with the protein Cdc42 playing a major role from budding yeast to human cells[3]. In *S.cerevisiae*, this GTPase manipulates the cell shape by regulating processes ranging from vesicular trafficking to actin cytoskeleton dynamics, septin deposition and mating[3–6]. Cdc42 promotes symmetry breaking in early G1 to produce a bud from an otherwise round cell. Initially, GTP-bound Cdc42 forms a polar cap and, after bud emergence, Cdc42 clustered activity at the bud tip directs growth of the daughter cell manipulating the actin cytoskeleton. At the end of mitosis, Cdc42 activity drops to allow cytokinesis[7].

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 tip[8–12]. Bnr1 and Bni1 recruit Bud6, an actin nucleation promoting factor, at sites of actin cables synthesis[13,14]. Bud6 enhances the actin nucleation activity of formins and regulates the early pathway of nuclear segregation[13–15]. 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 distribution[16]. 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[17–20]) are present in this organism. The only known GEF for Cdc42 in this organism is the essential protein Cdc24, which orchestrates the accumulation of GTP-Cdc42 to differentially localized clusters during the cell cycle[21,22]. 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[23,24]. Recruitment of Cdc24 at the plasma membrane (PM) in G1 relies on its physical interaction with Rsr1, a Ras-family GTPase, and with the Bem1 scaffold protein. Moreover, it was recently shown that Cdc28- and Cla4-dependent phosphorylation of Cdc24 regulates the activity and localization of the GEF, with non-phosphorylated forms accumulating at the bud tip[25]. These phosphorylation events occur mainly during mitosis, but a portion of the GEF remains phosphorylated also in early stages of the cell cycle[25]. Clustered Cdc24 is responsible for the local activation of Cdc42 and is an absolute prerequisite for *S.cerevisiae* cells to bud[26]. Interestingly, the few *rsr1*Δ*bem1*Δ surviving cells are, to some extent, still able to polarize, indicating the existence of yet another player[27–29].

Work in other organisms suggested the existence of a physical interaction between Cdc24 and active-Ras[30,31]. The physiological significance of this interaction and the mechanistic details underlying have not been investigated. 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 cancers[32]. 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 the PKA[33,34]. Ras exerts its essential role upon accumulation on the PM which is achieved through a secretory apparatus-dependent and a secretion-independent pathways[35–38]. Activity of Ras in *S.cerevisiae* 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[39–45]. Beside its essential role in G1, some observations for Ras activity in mitosis have been reported in budding yeast and other organisms[46–49]. The mechanistic details of the role of Ras in regulating Cdc42 are lacking and the impact of Ras on Cdc24 has not been investigated in detail[30,31].

The atypical protein-kinase haspin is conserved in 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[50,51]. 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 chromatids[52–59]. Recently H3-Thr3 phosphorylation has also been found to regulate asymmetrical histone inheritance in *Drosophila* male germline[60]. In budding yeast, two haspin paralogues, Alk1 and Alk2, have been

identified[61]. 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 bud[62]. We hypothesized that an altered regulation of polarization may be responsible for these phenotypes[62].

In this work, we analyzed the involvement of *S.cerevisiae* haspin in polarization dispersion. Our findings confirm that mislocalization of Bud6, which in *alk1*Δ*alk2*Δ cells is hyper-polarized to the bud tip and is missing from the bud neck, is a critical defect causing actin asymmetric distribution and asymmetric nuclear division. We show that yeast haspin ultimately regulates Cdc42, the master player of polarization. This function is exerted by modulating the recruitment of Cdc24, the Cdc42 GEF, whose localization we demonstrate to be regulated by Ras. The actual step promoted by haspin is a shift from a preferentially bud-tip directed to a uniform vesicle delivery to the daughter PM. 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[63].

RESULTS

Haspin modulates GTP-Cdc42 by regulating its proper distribution

In budding yeast cells that experience a mitotic delay, loss of haspin leads to the accumulation of actin and nuclear missegregation within the daughter cell[62]. This phenotype was suggested to be the consequence of an excessive accumulation of polarity factors at the bud tip, and particularly by the hyper-accumulation of Bud6 at this region and to its absence from the bud neck[62].

Failure to localize Bud6 at the bud neck has been related to defective activation of the Cdc42 GTPase at the same site[64,65]. Moreover, inactivation of Cdc42 results in a disorganized actin cytoskeleton, similarly to what observed after mitotic arrest in cells lacking haspin[16,62]. We thus hypothesized that loss of haspin may lead to defects in Cdc42 activation. This was initially tested verifying whether overexpression of *CDC42* rescued the phenotypes of *alk1*Δ*alk2*Δ cells. As shown in Figure 1a (for cell cycle analysis refer to Supplementary Figure 1a), induction of GAL-*CDC42* fails to restore a proper nuclear segregation in *alk1*Δ*alk2*Δ cells. If haspin is required for the local activation of Cdc42, increasing the protein level may not be sufficient to recover the distribution of GTPase activity, explaining the failure to alleviate the phenotype of *alk1Δalk2Δ* cells. We thus expressed the constitutively active *CDC42-G12V* allele, which indeed

suppresses the nuclear segregation defect of haspin-lacking cells, reducing it to the background level observed in wt cells expressing Cdc42-G12V[66]. Similarly, hyper-active Cdc42 restores the proper localization of Bud6, which is unaffected by elevated levels of wt Cdc42 (Figure 1b). These results indicate that haspin activity is crucial to promote localization of active Cdc42 at the bud neck or to locally activate Cdc42, supporting the model proposed by Panigada et al[62].

To verify this model, we used a fluorescent probe constituted by a CRIB-TdTomato chimera that binds to GTP-loaded Cdc42 allowing to specifically evaluate the localization of the active form of Cdc42[7,67,68]. The probe was expressed in wt and *alk1*Δ*alk2*Δ strains and, following an M-phase delay, cells were analyzed by fluorescence microscopy. Control cells show a generally homogenous distribution of GTP-loaded Cdc42, even though in a fraction of them some accumulation of active Cdc42 at the bud tip is also detectable (Figure 1c-e and Supplementary Figure 1b). In the absence of haspin, on the other hand, active Cdc42 is largely recruited at the bud tips (85% cells; Figure 1c-e); no significant localization of active Cdc42 is observed along the rest of the PM. This was further proved by measuring 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. Consistent with the rest of the data, this value is significantly higher in *alk1*Δ*alk2*Δ cells compared to wt counterparts (Supplementary Figure 1c).

Altogether, these results suggest that loss of haspin confines active Cdc42 to the bud tip, which leads to mislocalization of key polarity factors.

Localization of polarity factors is a dynamic process and is followed by dispersion of the polarized proteins. Indeed, Cdc42 is known to be hyper-polarized at the bud tip in G1 and is later redistributed throughout the cell. The phenotype described above may thus result from hyper-accumulation of Cdc42 activity at the bud tip or from a failure to disperse the polarity cap.

Haspin and Ras regulate Cdc24 localization

As suggested by Figure 1, we hypothesised that the role of haspin in modulating Cdc42 may be to control proper localization of GTP-Cdc42 along the PM.

The activity of Cdc42 in budding yeast is regulated positively by a single, essential GEF, Cdc24[17–22]. In particular, precise localization of Cdc24 is crucial to locally activate Cdc42. We thus investigated whether haspin may affect the localization of Cdc24.

Wt and *alk1Δalk2Δ* cells expressing GFP-Cdc24 were pre-synchronized in G1 and released in nocodazole-containing medium; 2.5 hours after the release, we monitored the localization of the GEF. In wt cells, most Cdc24 is found all over the PM, consistently with the homogenous distribution of active Cdc42 at the cortex. In contrast, in absence of haspin, Cdc24 is strongly accumulated at the bud tip, explaining the elevated levels of

active Cdc42 at the same location (Figure 2a-c and Supplementary Figure 2a). We previously reported that in *alk1*Δ*alk2*Δ cells some polarity factors are mislocalized also in unperturbed cycling cells[62]. An obvious prediction, if Cdc42-GTP resilience at the bud tip is the leading cause for the defects of *alk1*Δ*alk2*Δ cells, would then be that haspin mutants should be defective for Cdc24 localization also in unperturbed conditions. We then monitored the distribution of the GEF in wt or haspin lacking cells during a G1-G1 cell cycle taking samples every 10' to monitor the localization of Cdc24. As expected, *alk1*Δ*alk2*Δ cells showed a more persistent accumulation of the GEF at the bud tip following G1 synchronization and release (Figure 2a, 2d and Supplementary Figure 2b).

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 Rsr1[27]. The observation that *RSR1* deletion could not rescue Cdc24 mislocalization in haspin mutants (Supplementary Figure 2c), indicates that Rsr1 is not critical for regulating the GEF during mitosis, suggesting that other factors may account for Cdc24 localization in this phase.

A direct physical interaction between active Ras and Cdc24 has been previously reported in other organisms, although its functional significance has not been determined[30,31]. Budding yeast genome encodes two Ras paralogues, Ras1 and Ras2. Viable cells carrying the double deletion 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 *alk1*Δ*alk2*Δ cells carrying a *bcy1*Δ allele, and analysing Cdc24 distribution in nocodazole-arrested cultures. *BCY1* only plays a marginal role in Cdc24 distribution (Figure 2e). Deletion of *RAS1 RAS2* in wt cells lowers the amount of Cdc24 found on the cortex. Indeed, the normalized Cdc24 fluorescence intensity ratio between PM and cytoplasm is significantly decreased (Figure 2f, 1=homogeneous distribution between membrane and cytoplasm), indicating that Ras is relevant for Cdc24 distribution in mitosis. We also noticed a residual Cdc24 at the bud tip in wt cells lacking Ras, which is likely due to other factors playing a minor role in mitosis. Importantly, RAS is critical for Cdc24 localization also in *alk1*Δ*alk2*Δ mitotic cells. Indeed, while loss of haspin causes Cdc24 to accumulate at the bud tip (Figure 2e and Supplementary Figure 2d), removal of Ras in *alk1*Δ*alk2*Δ cells suppresses this phenotype, restoring Cdc24 distribution to that of a *ras1*Δ*ras2*Δ control.

Together, these results identify Ras as a critical factor to recruit Cdc24 to the PM. A possible interpretation is that Ras helps Cdc24 recruitment to the cell cortex, including the bud tip, and that haspin is critical to disperse the bud-tip bound fraction of Cdc24, eventually acting at the level of the GTPase. This mechanism 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 may have only marginal roles.

Active-Ras and Cdc24 physically interact

Previous works in *S.pombe* and *C.neoformans* identified a physical interaction between Cdc24 and GTP-loaded Ras by two-hybrid analyses[30,31]. An appealing hypothesis was hence that active-Ras was required for Cdc24 recruitment to the PM by direct physical interaction with the GEF.

We first tested whether Cdc24 and Ras physically interact in budding yeast performing a bimolecular fluorescence complementation (BiFC) between Cdc24-Venus^{C} and Venus^{N}-Ras2. We classified cells in unbudded, small budded or large-budded and scored the percentage of cells with fluorescent signal and its distribution. Control cells expressing either of the two constructs alone did not exhibit any measurable fluorescence. When both constructs were simultaneously expressed, as shown in Figure 3a, about half of single cells showed an even distribution of Cdc24-Ras2 complex along the PM. The percentage of fluorescence positive cells increased upon budding, with the majority of small-budded cells accumulating the complex only on the mother cortex, while a fraction showed a polarized signal at the bud tip. Finally, virtually all large-budded cells had a fluorescent signal both in mother and daughter cell, with a preferential accumulation of Cdc24-Ras2 along the whole PM and with a fraction of the population also showing fluorescence at the tip. Our data, not only demonstrate that Cdc24 and Ras2 interact in budding yeast, but they also indicate that such interaction is regulated temporally and spatially.

Reports by Yoshida and Geymonat identified an Lte1-Ras2 interaction, which requires a preliminary phosphorylation of Lte1 by Cla4 and Cdc28 to occur[47,69]. Intriguingly, Cdc24 was recently found to be phosphorylated by the same kinases and it was demonstrated that its phosphorylation promotes dispersion from the bud tip[25]. A similar mechanism may exist also for Cdc24-Ras2.

To test this, we introduced mEOS-tagged Cdc24, Cdc24-46A (in which 46 residues predicted targets of Cla4 or Cdc28 were mutated to alanine) or Cdc24-28D (in which 28 of these sites were mutated to aspartate) in wt cells and monitored distribution of the different GEF variants during mitotic delays. Figures 3b and 3c report the fraction of nocodazole arrested cells where the various Cdc24 mutants are polarized at the bud tip. Cdc24-mEOS, like Cdc24-GFP, was mostly homogeneously distributed in cells experiencing a mitotic delay (12% cells with polarized Cdc24-mEOS). As expected from the notion that in mitosis Cdc24 is normally phosphorylated by Cla4, the phospho-mimetic Cdc24-28D-mEOS also showed a homogenous distribution in our experimental setup. Intriguingly, the Cdc24-46A phospho-mutant was instead strongly accumulated to the bud tip, indicating that phosphorylation of Cdc24 is required in mitosis to redistribute the GEF from the bud tip to the whole cortex. This finding elicits the hypothesis that Cdc24 phosphorylation is a molecular switch that transfers Cdc24 from the bud tip polarized factors (Bem1 and Rsr1) to the PM distributed RAS. If this is true we can predict that,

upon removal of Ras, the phosphorylated Cdc24, although with lower affinity, may partly remain bound to the tip-localized polarity factors due to the absence of a competing partner at the cortex. This situation should be preserved in a Cdc24 phosphomimetic mutant. On the other hand, non-phosphorylatable mutant, which maintains a strong affinity for Rsr1 and Bem1 partners, should always be localized at the bud tip, irrespectively to the presence or absence of Ras. Its distribution should thus not differ between a wt and a *ras1*Δ*ras2*Δ*bcy1*Δ strain. These predictions were verified introducing the different *CDC24* alleles in a *ras1*Δ*ras2*Δ*bcy1*Δ background. Figure 3c shows that, in the absence of RAS, the distribution of the phosphomimic Cdc24-28D is the same as that of a wt Cdc24, and that the bud-tip localization of the the Cdc24-46A non-phosphorylatable form is not affected by loss of RAS.
Together, our results show that Cdc24 physically interacts with GTP-Ras2 and that this interaction is cell-cycle regulated through phosphorylation of Cdc24.

Haspin regulates localization of Ras

We have shown above that, in mitosis, haspin is required to delocalize GTP-Ras-recruited Cdc24 from the bud tip. To clarify the role played by haspin on Ras, we first committed to determine the impact of haspin loss on the levels of active-Ras. To this end, we exploited a GST-RBD (Ras Binding Domain from human Raf1) fusion, which specifically binds active Ras. We performed GST-RBD pulldown assays. As shown in Figure 4a, the amount of

active Ras2 is decreased in *alk1*Δ*alk2*Δ cells compared to a wild-type strain (for cell-cycle analysis and resin specificity refer to Supplementary Figure 4a-b respectively). The possibility that the defects observed in haspin-lacking cells may be due to the lower amount of Ras-GTP was excluded, as expressing a constitutively active *RAS2-G19V* allele did not rescue the nuclear missegregation defect of haspin-lacking cells (Supplementary Figure 4c).

To explain the Cdc24 hyper-polarization observed in *alk1*Δ*alk2*Δ mutants, we then reasoned that haspin may control the distribution of Ras activity. This possibility was confirmed monitoring the localization of the GFP-RBD probe, which specifically binds to GTP-loaded Ras[70–72]. After a mitotic delay, in cells lacking haspin, active Ras is strongly hyper-polarized towards the bud tip, while in control cells GTP-Ras is distributed throughout the plasma membrane of both mother and daughter cells (Figure 4b-d, Supplementary Figure 4e). This finding is confirmed by measuring the centre of mass-centroid distance, which is higher in haspin-lacking cells (Supplementary Figure 4f). Noteworthy, overexpression of either wt or constitutively active *CDC42* does not rescue this altered localization (Supplementary Figure 4g). The fact that overexpression of *CDC42-G12V* suppresses nuclear division and Bud6 distribution defects but not localization of GTP-Ras, confirms that the latter acts upstream of Cdc42.

To assess whether active-Ras localization is altered also in unperturbed conditions, in agreement with the persistent accumulation of Cdc24 at the tip upon loss of haspin, we synchronized wt and *alk1*Δ*alk2*Δ 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 4b-e and Supplementary Figure 4h). This result demonstrates that haspin is a critical factor that ensures a proper distribution of Ras activity in the cell. Failure to disperse 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 Sdc25[39–45]. Sdc25 is active only in particular nutrient conditions, we thus investigated the possibility that mislocalization of Cdc25 may be responsible for the altered distribution of active-Ras in haspin-defective cells[45]. As shown in

Supplementary Figure 5a, we found no differences in Cdc25 distribution in wt or *alk1*Δ*alk2*Δ cells during an M-phase delay, with the GEF accumulating at internal structures as previously reported[38,73]. If loss of haspin led to impairments in localization of the GAPs, then deletion of *IRA1* and *IRA2* may restore wt phenotypes. Supplementary Figure 5b shows that removal of Ras GAPs has only a minor attenuating effect on the nuclear and actin defects of *alk1*Δ*alk2*Δ 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 5a-b and Supplementary Figure 5d). A similar defect was observed through immunofluorescence on endogenous Ras2, excluding artefactual results due to *GFP-RAS2* overexpression (Supplementary Figure 5d). Together, these data indicate that in budding yeast haspin controls dispersal of Ras from the bud tip.

Ras regulates nuclear segregation in response to mitotic delays

We have shown that dispersal of polarity caps is critical for nuclear segregation after a mitotic delay, and that Ras2 plays a

major role in redistributing polarity factors during mitosis. We thus expect that *RAS* may regulate nuclear segregation upon delaying mitosis.

Actin distribution and nuclear segregation in nocodazole treated *ras1*Δ*ras2*Δ cells is defective (Figure 6a-b, Supplementary Figure 6), albeit only partially, as expected from the residual Cdc24 at the bud tip (Figure 2e). Noteworthy, both defects occurred preferentially in daughter *ras1*Δ*ras2*Δ*bcy1*Δ cells (Figure 6c-d), similarly to what happens upon haspin loss. In accordance with the impact of active Ras in modulating cell polarity and with the role of haspin in directing Ras localization, deletion of *RAS* restored nuclear missegregation and actin misdistribution of *alk1*Δ*alk2*Δ strains to that of a *ras1*Δ*ras2*Δ background (Figure 6a-b).

Haspin promotes isotropic vesicle-mediated Ras distribution to the PM during mitosis

Localization of Ras to the PM in budding yeast relies on two distinct pathways, a Erf2/Erf4 dependent mechanism, which promotes Ras palmitoylation, and a secretion dependent mechanism based on vesicular traffic. Loss of either one of the two branches of Ras localization does not prevent PM recruitment of the GTPase, while abrogation of both results in accumulation of the GTPase on endomembranes[36,37]. The defect in Ras2 distribution observed in haspin mutants could arise as a consequence of altered delivery routes of Ras-loaded vesicles. To

test this hypothesis, we constructed wt or *alk1*Δ*alk2*Δ strains bearing *pGAL-GFP-RAS2* and expressing the *sec6-4* allele, which, at restrictive temperature, impairs vesicle tethering to the PM. If loss of haspin led to Ras accumulation at the bud tip due to preferential vesicular traffic toward that district, blocking trafficking through the *sec6-4* mutation, should rescue, at least partially, Ras hyper-polarization in *alk1*Δ*alk2*Δ cells. Diffused PM localization of GFP-Ras2 would anyway be guaranteed thanks to the Erf2-mediated pathway. Strikingly, Figure 7a shows that inactivation of Sec6 did not alter the localization of Ras2 in otherwise wt cells, while it completely abolished the localization defect of *alk1*Δ*alk2*Δ cells. This finding demonstrates that haspin is needed for isotropic RAS distribution along the cortex through vesicles. Noteworthy, inactivation of exocytosis caused the accumulation of GFP-Ras2 in discrete dots, likely secretory vesicles, either along the PM or dispersed in the cytoplasm, supporting the notion that Ras reaches the PM in a vesicle-mediated manner.

Our data raised the possibility that defective distribution of Ras in haspin mutants actually stems from defective secretory routes in these cells. To test this hypothesis we introduced a GFP-Snc1 construct in wt or *alk1*Δ*alk2*Δ cells. We report, indeed, that loss of haspin caused a persistent accumulation of the SNARE at the bud tip, both following a M-phase arrest and in an unperturbed cell-cycle (Figure 7b-d and Supplementary figure 7b). Given the established interplay between Cdc42, actin cytoskeleton and exocytosis, we could not absolutely exclude the hypothesis that

preferentially tip-directed vesicular traffic could be ascribed to the hyper-polarization caused by loss of Alk1 and Alk2. This possibility is however unlikely since deletion of *RAS1 RAS2,* suppressed the hyper-polarization of Cdc24 in haspin mutants (Figure 2e), while it did not rescue the accumulation of Snc1 at the tip, as shown in Supplementary Figure 7c.

Overall, we describe a novel regulatory axis that controls the dispersal of polarization-promoting factors, through the regulation of exocytic routes of Ras-containing vesicles mediated by haspin kinase. In budding yeast this pathway is critical to tolerate mitotic delays and we predict that it may be significantly relevant also in higher organisms.

DISCUSSION

Control of cell polarity is critical for development, organ and tissue function and differentiation. Its alteration is linked to pathologies and carcinogenesis, making understanding the bases for polarity regulation a key challenge. Budding yeast *Saccharomyces cerevisiae* has proven to be an invaluable tool to dissect polarity onset and the function 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 deal with polarity dispersion and what are the

consequences of a failure in such process. Previously, we provided a first insight on the effects of a prolonged polarization on budding yeast cells. We reported that *alk1*Δ*alk2*Δ cells, lacking haspin kinase paralogues, accumulate excessive polarity factors at the bud tip, and after an M-phase delay this causes actin accumulation in the daughter cell, nuclear missegregation and ultimately cell death[62]. Here we shed light on the mechanisms underlying haspin function, unveiling its role in polarisome dispersion, through modulating Ras distribution, and showing that timely relocalization of polarity proteins is a fundamental event in the cell cycle.

In our previous work, we reported that the actin nucleation-promoting factor Bud6 is mislocalized in haspin-lacking cells, where it accumulates at the bud tip and is missing from the bud neck. We proposed this to be the leading cause for actin and nuclear segregation defects in such cells.

Bud6 is an effector of the small GTPase Cdc42, the master regulator of polarization in all eukaryotes, and impairments in Cdc42 result in the building of non-properly organised actin networks in budding yeast[16]. Cdc42 has also been shown to regulate both actin and nuclear segregation in human cells, making it an appealing candidate for haspin-dependent regulation[74]. Overexpression of a hyper-active allele of the GTPase, but not of its wt counterpart, is sufficient to recover the phenotypes of haspin-lacking cells in terms of Bud6 localization and nuclear segregation (Figure 1a-b). This strongly suggested

that *alk1*Δ*alk2*Δ cells may be defective in the distribution of active Cdc42 rather than that of the total Cdc42 population. Indeed, in the absence of haspin GTP-Cdc42 was accumulated at the bud tip compared to wt control cells (Figure 1c-e and Supplementary Figure 1).

Cdc42 is activated through GTP loading by its GEF, Cdc24. We then analyzed the distribution of Cdc24. In mitotic wt cells, Cdc24 was dispersed all over the cell membrane, reflecting the homogenous distribution of GTP-Cdc42. Conversely in the absence of haspin, Cdc24 was mostly found at the bud tip, explaining the accumulation of GTP-Cdc42 in the same region (Figure 2a-c and Supplementary Figure 2a). A similar defect was observed even in unperturbed synchronous cells, where loss of Alk1 and Alk2 caused a stronger persistence of the GEF at the bud tip (Figure 2d). This result is particularly relevant as it provides a timing mechanism for polarisome dispersion during M-phase: if cells progress efficiently through mitosis, Cdc24 accumulation at the bud tip, being only temporary, does not lead to any severe effect. On the other hand, if cells experience a mitotic delay, clusters of active Cdc42 at the bud tip need to be readily dispersed through the action of haspin. Failure to remove these clusters triggers a cascade of perturbations in protein localization that ultimately results in nuclear missegregation and cell death.

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, however *RSR1* deletion had no impact on Cdc24 localization in mitotic cells (Supplementary Figure 2c), suggesting the existence of distinctive recruitment mechanisms for the GEF that act specifically in different stages of the cell-cycle. Hints for a physical interaction between budding yeast GTP-Ras and Cdc24 derive from works in other organisms, and suggest that Ras may play a relevant role in regulating Cdc24[30,31]. Deletion of Ras-coding genes noticeably decreased the recruitment of Cdc24 to the plasma membrane in wt M-phase arrested cells (Figure 2e), supporting the hypothesis. The small increase in the percentage of cells with polarized Cdc24 observed in *ras1*Δ*ras2*Δ cells is likely due to the small fraction of Cdc24 retained at the bud tip in this mutant, through other pathways (e.g. Rsr1). Strikingly, removal of RAS from *alk1*Δ *alk2*Δ cells suppressed the accumulation of Cdc24 at the bud tip caused by loss of haspin (Figure 6a-b). These results further suggested that Cdc24 distribution during mitosis relies on a direct recruitment by Ras.

Through BiFC analyses, we demonstrate an *in vivo* physical interaction between Ras2 and Cdc24 in budding yeast, and studied its spatio-temporal regulation. Indeed, we report that such interaction is restricted to the mother PM early in the cell-cycle and then it is promoted also in the daughter cell, where it first occurs at the bud tip and then is redistributed towards the whole membrane (Figure 3a). This result, together with previous reports on how Cdc24 is recruited 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. We however propose that Ras-dependent mechanism for Cdc24 PM recruitment may account for the reported capability of some *rsr1*Δ*bem1*Δ strains to successfully polarize Cdc24[27–29]. This is not the only protein relocalizing from the bud tip to the PM in a Ras-dependent manner. Works by Yoshida et al. and Geymonat et al. showed that Lte1, which initially accumulates at the bud tip, is recruited during mitosis to the PM following its binding to GTP-Ras[47,69]. The change in Lte1 interactors is promoted by a series of phosphorylation events mediated by Cdc28 and the PAK Cla4.

Intriguingly, Cdc24 was recently reported to be subjected to cell cycle dependent phosphorylations from CDK and Cla4. These posttranslational modifications regulate Cdc24 distribution and activity in the cell: non-phosphorylatable forms of Cdc24 cause its accumulation at the bud tip[25]. We show that in mitosis, a stage where Cdc24 should be evenly distributed to the whole PM, a phosphomutant form is instead still restricted to the bud tip (Figure 3b). This observation is consistent with the hypothesis that phosphorylation of the GEF promotes a shift in Cdc24 interactors, releasing it from its G1 partners (e.g. Rsr1) to favour binding to mitotic ones (i.e Ras). Failure to phosphorylate Cdc24 would then result in the persistence of its interaction with its G1 partners also later in the cell cycle. Indeed, loss of Ras did not affect the hyper-polarization observed when Cdc24 is non-phosphorylatable,

suggesting that the non-phosphorylated GEF relies on partners other than Ras for its localization.
We suggest that, as cell cycle proceeds, phosphorylation of Cdc24 would promote a switch between partners where GTP-Ras substitutes Rsr1, resulting in redistribution of Cdc24, followed by GTP-Cdc42, from the bud tip to the whole PM.

Loss of haspin has a bivalent effect on GTP-Ras in mitotic cells: on one hand, it causes a generalized decrease in the levels of active Ras, while, on the other hand, it alters the distribution of active GTPase by manipulating the localization of the whole pool of Ras (Figure 4a and 5). Though we have not extensively investigated the outcome of the global reduction of active-Ras, we showed that effective dispersion of Cdc24 from the bud tip stems from modulating the localization of GTP-Ras rather than its levels. The defective distribution of Ras observed in *alk1*Δ*alk2*Δ cells was recovered by inactivation of the exocytic pathway, and analysis of vesicular traffic revealed that haspin-lacking cells do not evenly distribute secretory vesicles towards the whole PM and instead direct them toward the bud tip.

Overall, 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 exocytic routes promoting a relocalization of GTP-loaded Ras that in turn recruits Cdc24 along the PM through physical interactions. This shift in the pattern of Cdc24 is required to allow redistribution

of GTP-Cdc42 as schematically represented in Figure 8. This work provides, to the best of our knowledge, the first mechanistic insights on how the depolarization process is promoted.

How haspin regulates vesicle delivery is still not clear. However, we can speculate that the most likely events leading to the observed hyper-polarization could be related to local landmarks that mediate vesicle tethering to the PM, such as the distribution of membrane-bound exocyst components. This aspect of haspin function will need further experimental analysis.

Given the extreme conservation of the proteins involved, we propose that this regulatory pathway may be conserved in all eukaryotes to regulate polarization-driven processes. Deregulation of this network may be responsible for the reported defects observed in *A.thaliana* haspin mutants, where the plane of the first cell division is skewed, the pattern of the following divisions is changed and vascular patterning is aberrant.[63]

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 described[75]. Standard molecular genetics techniques were used to construct plasmids and strains. The centromeric plasmids containing, *GFP-3RBD, CDC25-eGFP* and *GST-RBD* were kind gifts of Dr. E.Martegani[72], that coding for *GST* was

kindly provided by Dr. D.Pellman. *GFP-BUD6* and *CDC24-eGFP* bearing strains were obtained transforming cells with pRB2190 and pYS37 respectively[76,77]. CRIB-TdTomato was kindly provided by Dr. DJ.Lew[68]. Plasmids and strains encoding for Cdc24 alleles were a kind gift of Dr. D.McCusker[25]. 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 strains used are isogenic to W303.

NAME	RELEVANT GENOTYPE	SOURCE
K699	*ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 MATa*	K.Nasmyth
yAN33	*alk1::NATr alk2::HIS3 MATa*	This work
yRQ315	*[GFP-BUD6][pGAL-CDC42] MATa*	This work
yRQ316	*alk1::NATr alk2::HIS3[GFP-BUD6][pGAL-CDC42] MATa*	This work
yRQ317	*[GFP-BUD6][pGAL-CDC42-G12V] MATa*	This work
yRQ318	*alk1::NATr alk2::HIS3 [GFP-BUD6][pGAL-CDC42-G12V] MATa*	This work
yRQ301	*CRIB-TdTomato-KANr MATa*	This work
yRQ302	*alk1::NATr alk2::HIS3 CRIB-TdTomato-KANr MATa*	This work

yRQ100	*[CDC24-GFP] MATa*	This work
yRQ101	*alk1::NATr alk2::HIS3 [CDC24-GFP] MATa*	This work
yRQ342	*rsr1::KANr [CDC24-GFP] MATa*	This work
yRQ343	*alk1::NATr alk2::HIS3 rsr1::KANr [CDC24-GFP] MATa*	This work
yRQ366	*bcy1::KANr [CDC24-GFP] MATa*	This work
yRQ367	*alk1::NATr alk2::HIS3 bcy1::KANr [CDC24-GFP] MATa*	This work
yRQ368	*ras1::TRP1 ras2::HPHr bcy1::KANr [CDC24-GFP] MATa*	This work
yRQ369	*alk1::NATr alk2::HIS3 ras1::TRP1 ras2::HPHr bcy1::KANr [CDC24-GFP] MATa*	This work
yRQ418	*CDC24-VENUS^{Cterm}-TRP1 MATa*	This work
yRQ427	*HIS3-pGAL1-VENUS^{Nterm}-RAS2 MATa*	This work
yRQ428	*CDC24-VENUS^{Cterm}-TRP1 HIS3-pGAL1-VENUS^{Nterm}-RAS2 MATa*	This work
yRQ430	*alk1::NATr alk2::KANr CDC24-VENUS^{Cterm}-TRP1 HIS3-pGAL1-VENUS^{Nterm}-RAS2 MATa*	This work
yRQ451	*[CDC24-mEOS] MATa*	This work
yRQ452	*[CDC24-46A-mEOS] MATa*	This work
yRQ453	*[CDC24-28D-mEOS] MATa*	This work
yRQ454	*alk1::NATr alk2::HIS3 [CDC24-mEOS] MATa*	This work

yRQ455	*alk1::NATr alk2::HIS3 [CDC24-46A-mEOS] MATa*	This work
yRQ456	*alk1::NATr alk2::HIS3 [CDC24-28D-mEOS] MATa*	This work
yRQ462	*ras1::TRP1 ras2::HPHr bcy1::KANr [CDC24-mEOS] MATa*	This work
yRQ463	*ras1::TRP1 ras2::HPHr bcy1::KANr [CDC24-46A-mEOS] MATa*	This work
yRQ464	*ras1::TRP1 ras2::HPHr bcy1::KANr [CDC24-28D-mEOS] MATa*	This work
yRQ116	*ras2::TRP1 [RAS2] MATa*	This work
yRQ117	*alk1::NATr alk2::HIS3 ras2::TRP1 [RAS2] MATa*	This work
yRQ119	*ras2::TRP1 [RAS2-G19V] MATa*	This work
yRQ120	*alk1::NATr alk2::HIS3 ras2::TRP1 [RAS2-G19V] MATa*	This work
yRQ73	*[GFP-RBD3] MATa*	This work
yRQ74	*alk1::NATr alk2::HIS3 [GFP-RBD3] MATa*	This work
yRQ412	*ras1::TRP1 ras2::HPHr bcy1::KANr [GFP-RBD3] MATa*	This work
yRQ262	*[GFP-RBD3][GAL-CDC42] MATa*	This work
yRQ263	*alk1::NATr alk2::HIS3 [GFP-RBD3][GAL-CDC42] MATa*	This work
yRQ264	*[GFP-RBD3][GAL-CDC42-G12V] MATa*	This work
yRQ265	*alk1::NATr alk2::HIS3 [GFP-RBD3][GAL-*	This work

	CDC42-G12V] MATa	
yRQ84	*[CDC25-GFP] MATa*	This work
yRQ85	*alk1::NATr alk2::HIS3 [CDC25-GFP] MATa*	This work
yRQ93	*ira1::LEU2 ira2::URA3 MATa*	This work
yRQ95	*alk1::NATr alk2::HIS3 ira1::LEU2 ira2::URA3 MATa*	This work
yRQ358	*ras1::TRP1 ras2::HPHr bcy1::KANr MATa*	This work
yRQ359	*alk1::NATr alk2::HIS3 ras1::TRP1 ras2::HPHr bcy1::KANr MATa*	This work
yRQ409	*sec6-4 [pGAL-GFP-RAS2] MATa*	This work
yRQ410	*alk1::NATr alk2::HIS3 sec6-4 [pGAL-GFP-RAS2] MATa*	This work
yRQ197	*[GFP-SNC1] MATa*	This work
yRQ198	*alk1::NATr alk2::HIS3 [GFP-SNC1] MATa*	This work
yRQ444	*ras1::TRP1 ras2::HPHr bcy1::KANr [GFP-SNC1] MATa*	This work
yRQ445	*alk1::NATr alk2::HIS3 ras1::TRP1 ras2::HPHr bcy1::KANr [GFP-SNC1] MATa*	This work

Plasmids used in this work

NAME	RELEVANT GENOTYPE	SOURCE
pRQ24	*pRS314-pGAL1-CDC42*	This work
pRQ25	*pRS314-pGAL1-CDC42-G12V*	This work
pRB2190	*pACT1-GFP-BUD6*	D.Botstein[76]
pYS37	*pRS315-CDC24-GFP*	M.Peter[77]
pSH18-34	*4LexAop-LacZ URA3*	This work
pDM700	*pRS416-pCYC1-CDC24-mEOS-HIS6*	D.McCusker[25]
pDM701	*pRS416-pCYC1-CDC24-46A-mEOS-HIS6*	D.McCusker[25]
pDM704	*pRS416-pCYC1-CDC24-28D-mEOS-HIS6*	D.McCusker[25]
pRQ12	*pRS316-pRAS2-RAS2*	This work
pRQ14	*pRS316-pRAS2-RAS2-G19V*	This work
PB1622	*pGEX-5X-1-GST*	D.Pellman[7]
pGEX2T-RBD	*pLac-GST-RBD*	E.Martegani[71]
pYX242-GFP-RBD	*pYX242-eGFP-3RBD*	E.Martegani[70]
yEPCDC25eGFP	*CDC25-GFP*	E.Martegani[78]
B828	*yEP55-RAS2-GFP*	R.J.Deschenes[79]
GFP-SNC1	*pRS315-p_{TPI1}-GFP-SNC1*	K.Tanaka[80]

Western blot

To analyse proteins during nocodazole treatment, cells were grown in YPD medium, synchronized in G1 with α-factor (2 μg/ml), and released in the presence of nocodazole (10 μg/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, 22C5D8 for Pgk1, sc-yC19 for Ras2), as previously described[81]. 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 PBS[62]. 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 cell area and circularity, statistical significance was determined with a T-test (see Supplementary Figure 9a). 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 analyzed daughters and the number of fractions respectively (for further details see Supplementary Figure 9b and c).

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

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.

Measurement of Ras-GTP levels

Quantification of active Ras in the cells was performed through a GST-RBD pulldown assay as previously described[71]. Briefly, 50ml of early log culture per strain were arrested in nocodazole, pelleted and freezed at -80°C. Samples were then resuspended in ice-cold lysis buffer (HEPES pH7.5 25mM, NaCl 150mM, Nonidet-P40 1%, Na-deoxycholate 0.25%, glycerol 10%, EDTA 1mM, DTT 0.5mM, Na_3VO_4 1mM supplemented with a Roche complete protease inhibitor tablet) and subjected to mechanical lysis.

Samples were clarified, normalized and incubated with 5µl of GST or GST-RBD loaded glutathione resin for 30' at 4°C. Samples were then washed 3 times with lysis buffer, transferred to new tubes, pelleted and boiled in Laemmly buffer for subsequent SDS-PAGE and western blot analysis.

Immunofluorescence

Cells of given strains were fixed with 3.7% formaldehyde for 15' at RT. After washing, cell wall was digested with Zymoliase and samples were then spotted on polylysine-covered slides. After saturation with BSA, cells were incubated O/N with primary antibody (sc 28549), washed and then incubated two hours with fluorophore-conjugated secondary antibody.

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.

Concanavalin A Staining

Cells grown in YPD were washed with PBS and resuspended in 125 µl of Alexa Fluor 488-conjugated concanavalin A (ThermoFisher C11252) at a concentration of 40 µg/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 1µM SytoxGreen and DNA content was determined using a FACScan cytofluorimeter.

ACKNOWLEDGMENTS

M. Peter, E. Martegani, D. Lew, D. McCusker and D. Botstein are acknowledged for donating plasmids and strains. M.M-F lab is supported by grants from AIRC (n.15631), Telethon (GGP15227), MIUR (PRIN) and a FIRC fellowship (R.Q.). We sincerely thank Rosella Visintin for useful discussions.

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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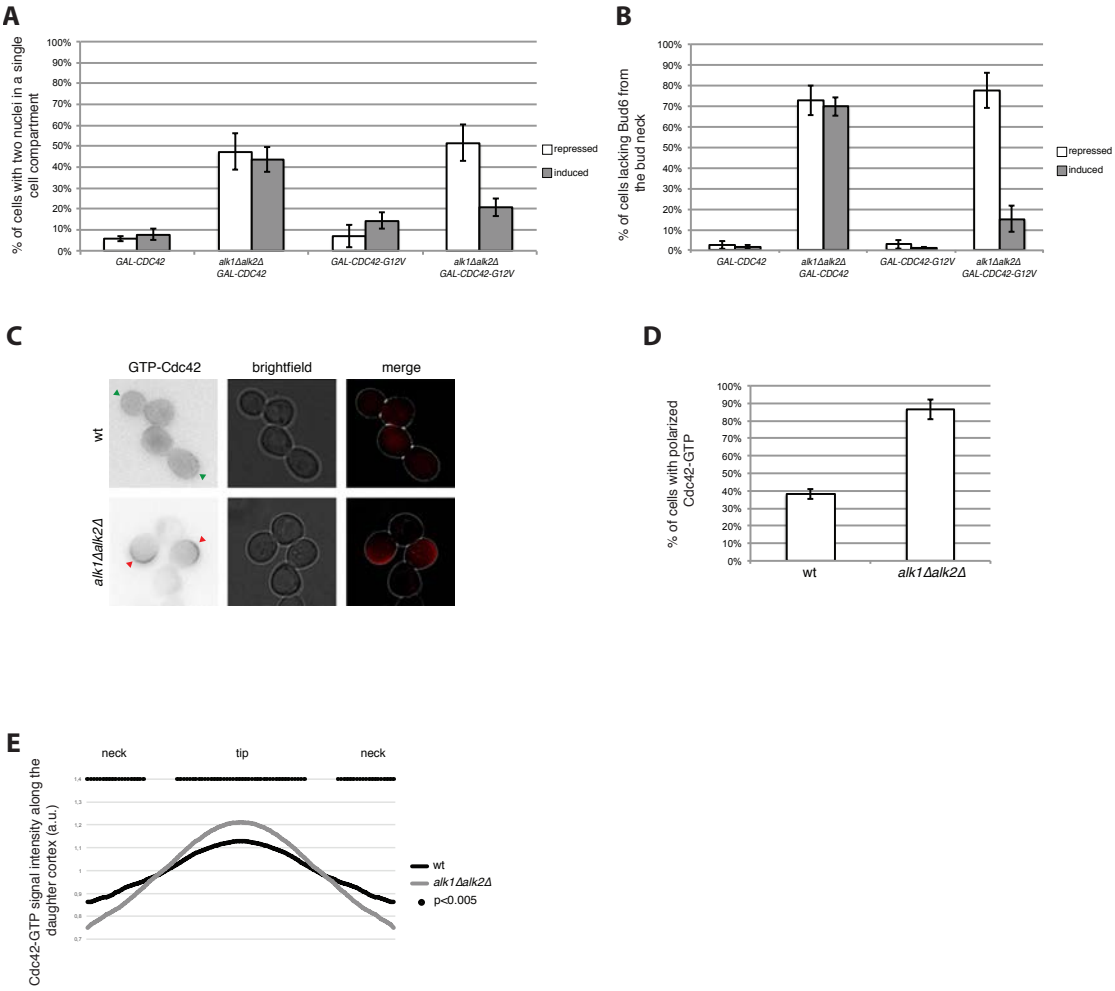
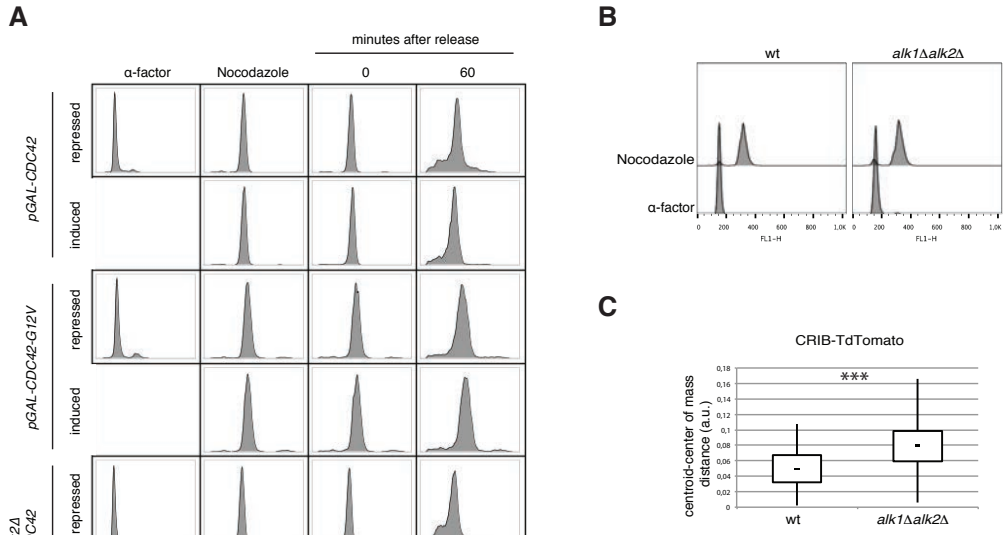


Figure 1 Haspin mediated regulation of Cdc42 activity is required for M-phase tolerance. wt or hyperactive Cdc42 was expressed in control or *alk1Δalk2Δ* cells under control of the GAL1 promoter and the effect of induction of the GTPase on nuclear segregation and Bud6 localization was assessed by fluorescence microscopy after an M-phase delay. Nuclei were stained with DAPI (**a**), Bud6 localization was detected by GFP-signal (**b**). (**c,d**) G1-arrested wt or haspin-lacking cells expressing CRIB-TdTomato (to assess localization of Cdc42-GTP) were released in nocodazole-containing medium for 2.5 hours and analysed by fluorescence microscopy, scoring the percentage of cells with polarized Cdc42-GTP; green and red arrows in panel c show cells with isotropic distribution of GTP-Cdc42 and sites of polarized Cdc42 activity respectively. The average CRIB-tomato signal intensity along the PM was quantified on 60 cells per strain (**e**), black dots represent fractions for which the intensity between the strains is significantly different (p<0.005).



Supplementary Figure 1 Panel **a** and **b** report the cell cycle arrests monitored by FACS analysis relative to the experiments in Figure 2a-b and c-e, respectively. The plot in **c** represents the centroid-center of fluorescence mass distance normalized on the daughter cell area and average daughter cell strain circularity, 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.

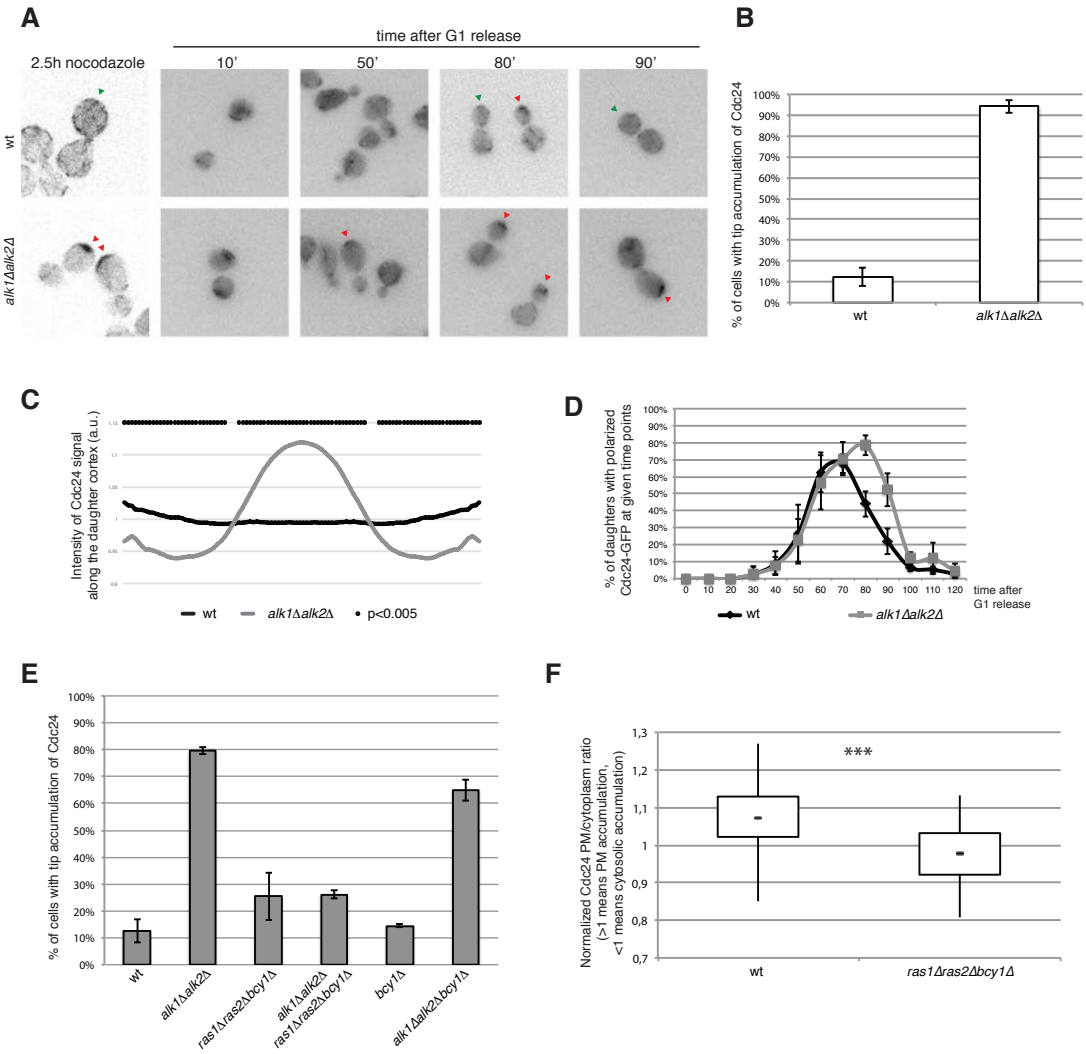
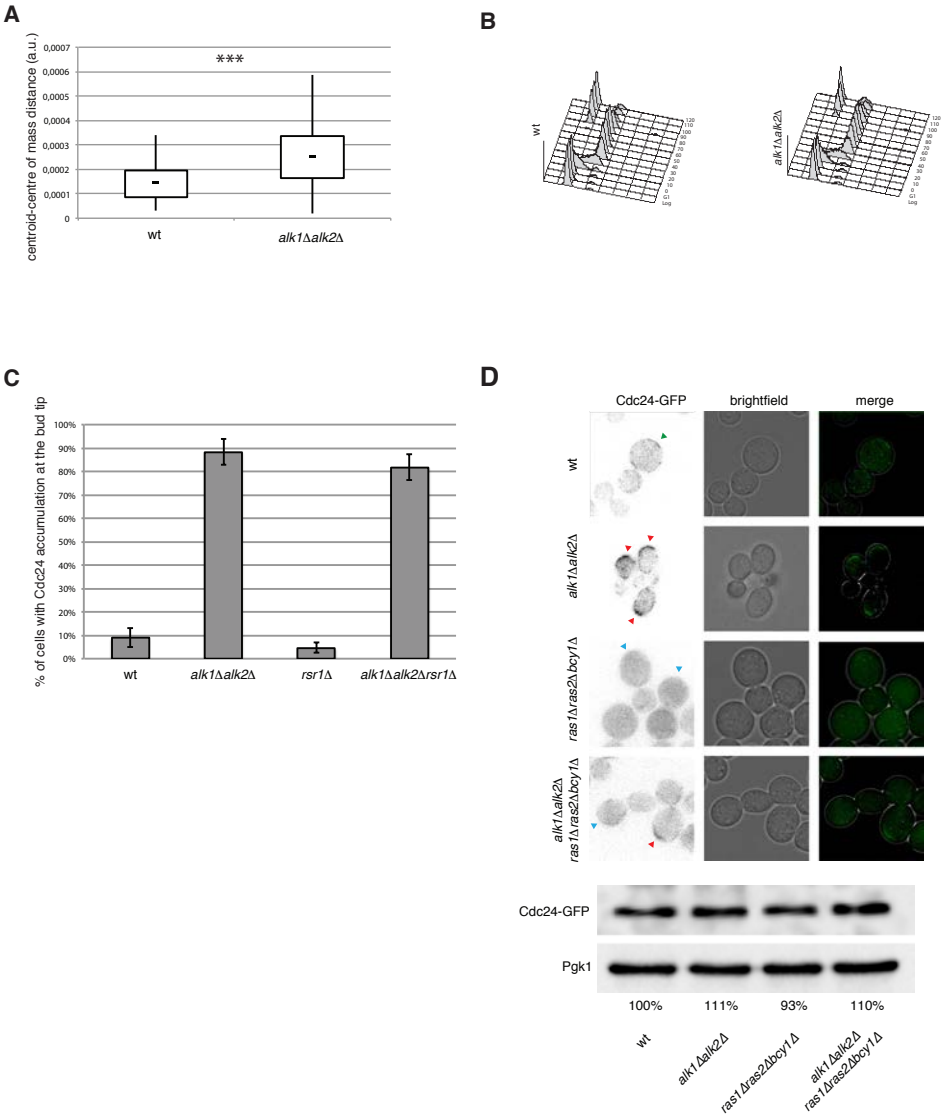


Figure 2 Localization of Cdc24 relies on haspin and Ras. **a**,**b**,**c**,**d** After presynchronization in G1, wild-type or haspin-lacking cells were arrested in nocodazole or released in drug-free medium and the localization of Cdc24-GFP was evaluated by fluorescence microscopy. Cells with accumulation of the GEF at the bud tip were scored at given time points (**a**; Green and red arrows in a indicate cells with PM localized Cdc24 and daughters with polarized Cdc24 respectively). at given time points, scoring cells with accumulation of the GEF at the bud tip at given time points (**b**) reports the percentage of cells with Cdc24 at the bud tip, 2.5 hours after G1 release in nocodazole-containing medium). The signal intensity along the PM was quantified and is reported in (**c**), black dots represent fractions for which the intensity between the strains is significantly different ($p<0.005$). The fraction of cells with Cdc24 at the bud tip during an unperturbed cell cycle is reported in **d**, error bars represent standard deviation). The plot in **e** represents the percentage of cells with polarized Cdc24 in given strains after 3 hours of nocodazole treatment. The graph in **f** 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, $p<0.001$.



Supplementary Figure 2 Graph in **a** shows the centroid-centre of mass distance, normalized on daughter area and daughter cell average strain circularity of 60 nocodazole arrested cells as in Figure 2a. Boxes include 50% of data points, line represents the average distance and whiskers report the minimum and maximum values. *** pvalue<0,001. Graph **b** shows cell-cycle progression monitored by FACS analysis for experiments described in Figure 3a-d. **c** is reports the percentage of cells of given strains with polarized Cdc24 after presynchronization in G1 and 2.5 hours of nocodazole treatment. Sample images of experiment described in Figure 3e and relative control of expression are shown in panel **d**; green, red and cyan arrows show cells with even Cdc24 distribution, sites of polarized GEF and cells with diffused Cdc24, respectively.

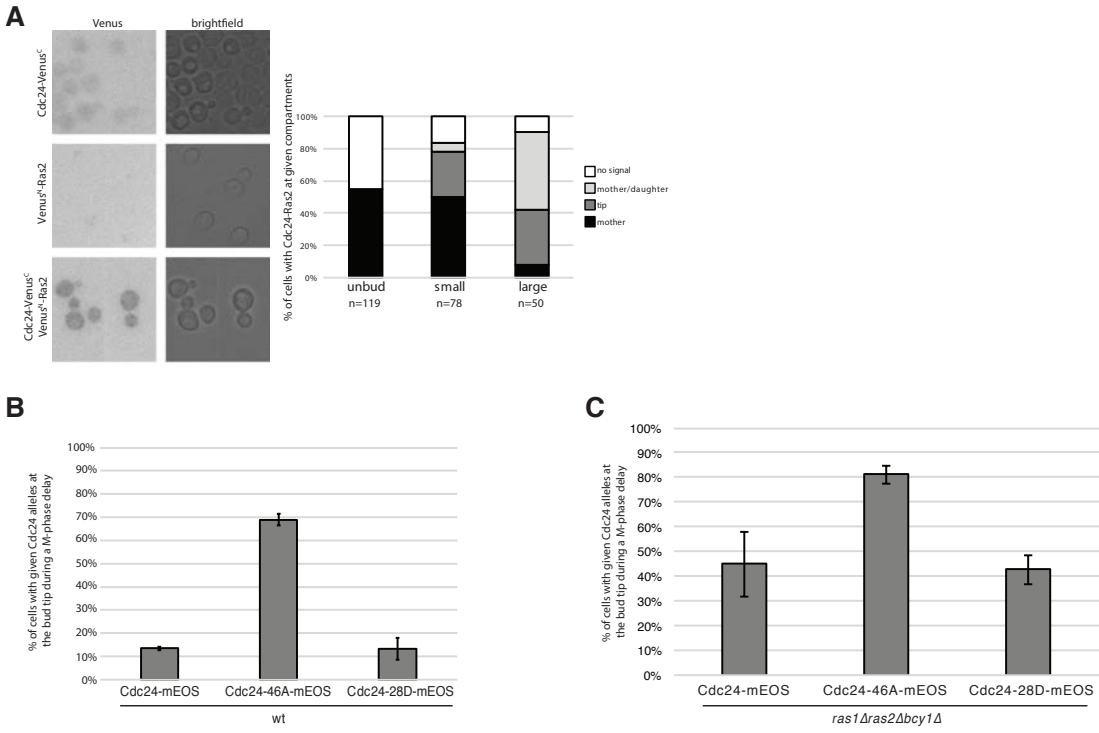


Figure 3 GTP-Ras and Cdc24 physically interact. **a** Cells of given strains were grown in raffinose containing medium and expression of Venus^{N}-Ras2 was induced by addition of 2% Gal for 2 hours. At the end of the induction the distribution of Venus signal was assessed. Quantification of cells where Venus signal is either absent, distributed along mother cortex, localized to both mother cortex and the bud tip or evenly accumulated along the PM of both cell compartments is reported on right panel. **b** wt cells expressing given Cdc24 alleles were arrested in nocodazole following G1 synchronization, fixed and analyzed by fluorescence microscopy to determine the distribution of the GEF. **c** Ras-deleted cells expressing given Cdc24 alleles were arrested in nocodazole for 3 hours, fixed and analyzed as above to determine the distribution of the GEF.

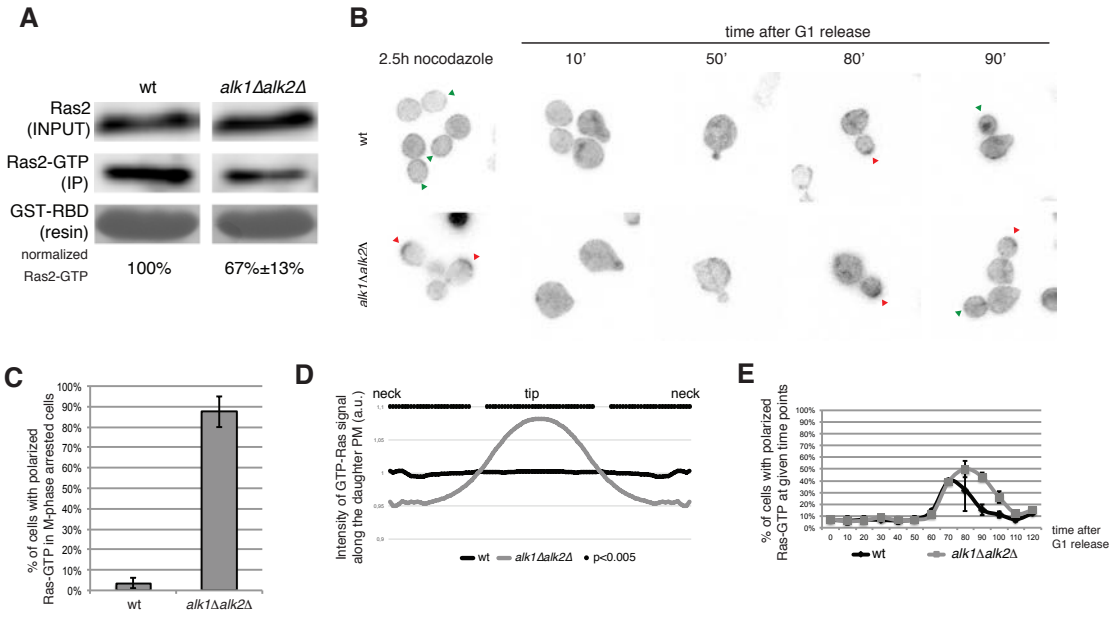
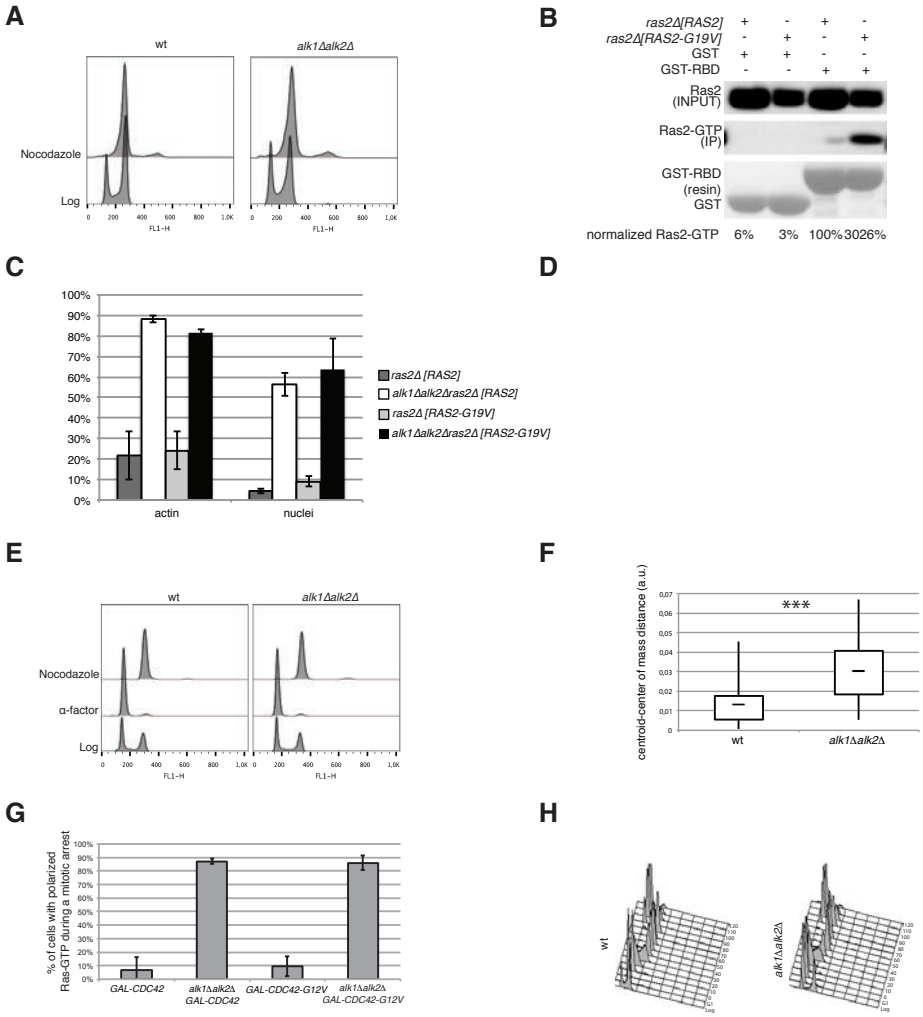


Figure 4 Haspin regulates active-Ras dynamics. **a** Ras2-GTP levels in nocodazole-arrested wt or haspin-lacking cells were estimated by pulling it down from crude protein extracts with GST-RBD and amount of precipitated protein were normalized to total Ras2. Average values and standard deviation are reported. The localization of Ras-GTP in wild-type or haspin-lacking cells was evaluated exploiting the GFP-RBD probe. Panel **b** shows examples of the images at given time points, green and red arrows show cell with diffused or polarized GTP-Ras2, respectively. Graphs **c** and **e** 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 **d**. Dots represent fractions with significant intensity difference.



Supplementary Figure 4 Plot **a** reports the cell-cycle arrest of the experiment in Figure 4a monitored by FACS analysis. Panel **b** reports a GST-RBD pulldown of Ras2-GTP performed on mitotically arrested *ras2Δ* cells complemented with given plasmid-coded *RAS2* alleles. To quantify Ras2-GTP, the level of purified protein was normalized on total Ras2 and GST-RBD amount of each sample. **c** Cells of given strains expressing either wt or constitutively active Ras2 were incubated for 3 hours in nocodazole-containing medium and then released, samples were taken at 0' or 60' after the release, respectively, to monitor actin distribution or nuclear segregation. Plot **e** shows the cell cycle arrests for experiment described in Figure 4c-d. Graph in **f** shows the centroid-centre of mass distance, normalized on daughter area and average daughter cell circularity, of 60 cells treated as in Figure 4c-d. Boxes include 50% of data points, line represents the average distance and whiskers report the minimum and maximum values. **g** cells of given strains expressing the *GFP-3RBD* construct were grown on raffinose, arrested in G1 and released in nocodazole-containing medium. 2 hours after the release, galactose was added to induce *CDC42* overexpression and the localization of active Ras was evaluated by fluorescence microscopy after another hour. Panel **h** shows the cell-cycle progression of experiment described in Figure 4e.

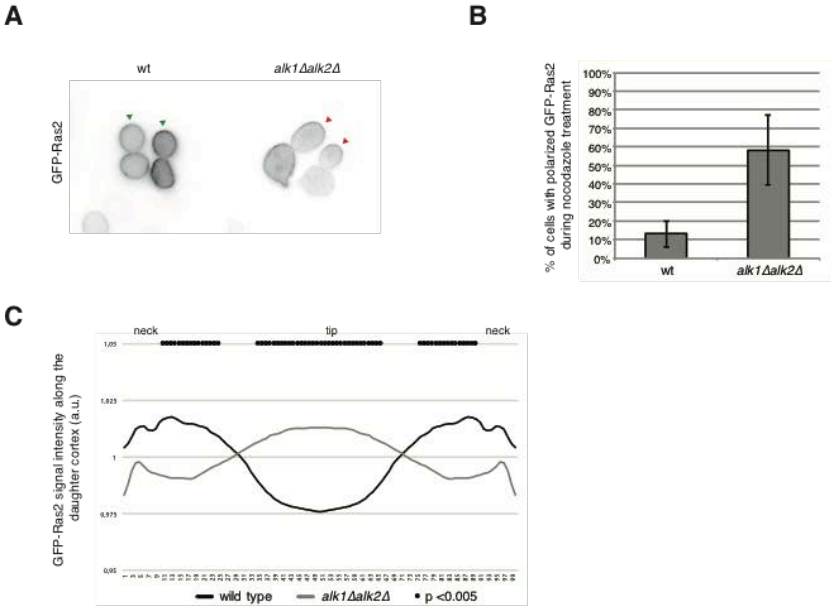
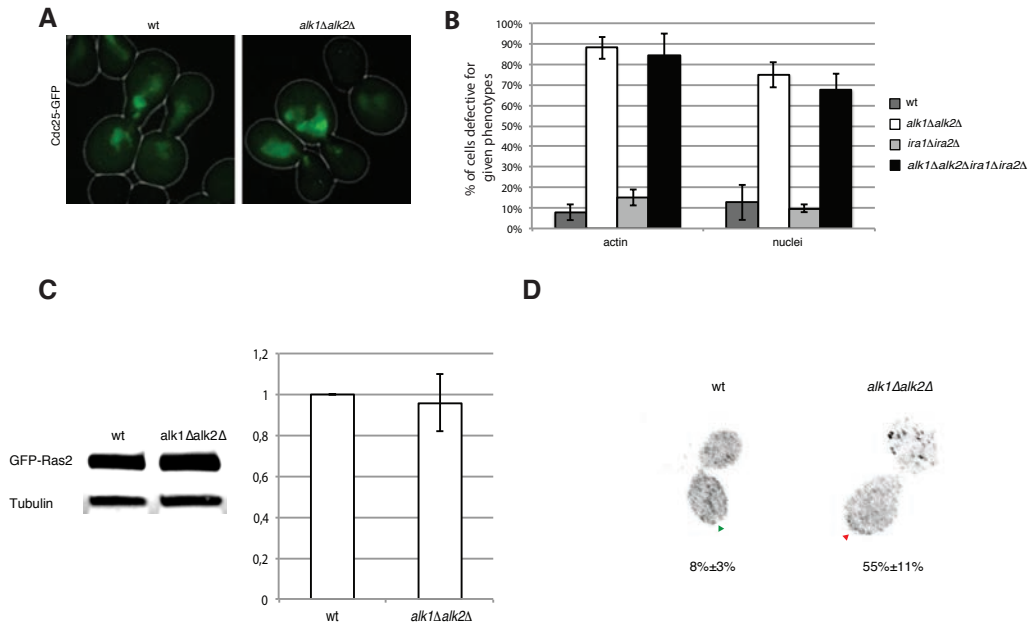


Figure 5 Localization of Ras is regulated by haspin. **a,b,c** Cells were arrested in G1 and then released for 2.5 hours in nocodazole. Galactose was added at the beginning of nocodazole treatment to induce expression of GFP-Ras. At the end of the treatment cells were fixed and the percentage of cells with polarized Ras2 were scored by fluorescence microscopy. Green and red arrows show cells with diffuse PM Ras2 or polarized GTPase, respectively, quantification of the phenotype is reported in **b**. Plot in **c** shows the signal intensity along the PM (black dots represent fractions for which the intensity between the strains is significantly different ($p<0.005$)).



Supplementary Figure 5 a-c Cells of the 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 scored by fluorescence microscopy. Panel **c** shows the induction control of GFP-RAS2 for the experiment in Figure 5. Picture in **d** shows sample images of Ras2 immunofluorescence in nocodazole-treated cells of given strains. Green and red arrows indicate cells with diffused PM Ras2 and polarized Ras2, respectively.

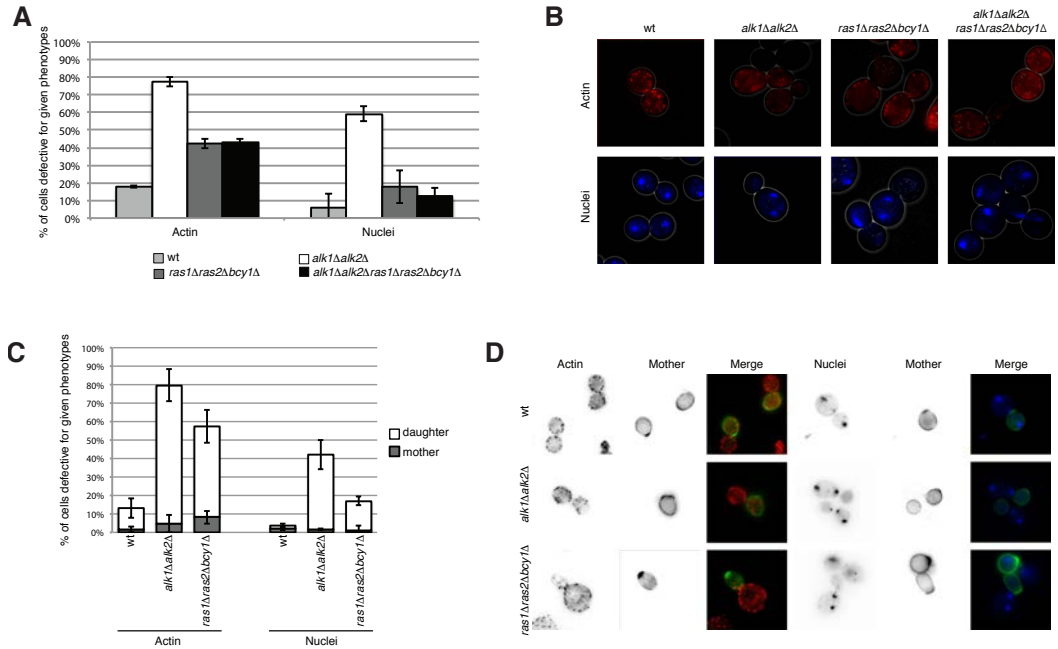
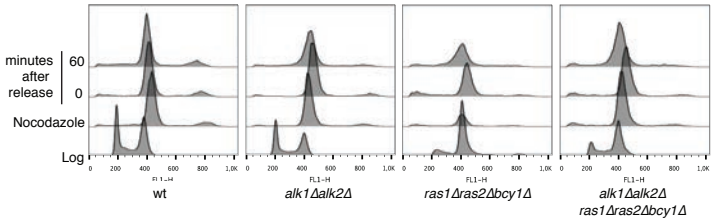


Figure 6 Ras is required for M-phase delay tolerance. **a,b** Cells of the indicated strains were treated for 3 hours with nocodazole. At the end of the treatment, the drug was washed out and actin (t=0' from the release) and nuclear segregation (t=60' from the release) were monitored by fluorescence microscopy as reported in material and methods. The percentage of cells exhibiting misaccumulated actin or missegregated nuclei is reported in **a**, while representative images are shown in **b**. **c,d** Cells were stained 10' with ConA-488, grown for an hour and then processed as in the previous experiment. Staining with ConA was exploited to distinguish mother and daughter cells.



Supplementary Figure 6 Cell-cycle analysis by FACS of experiment in Figure 7a,b

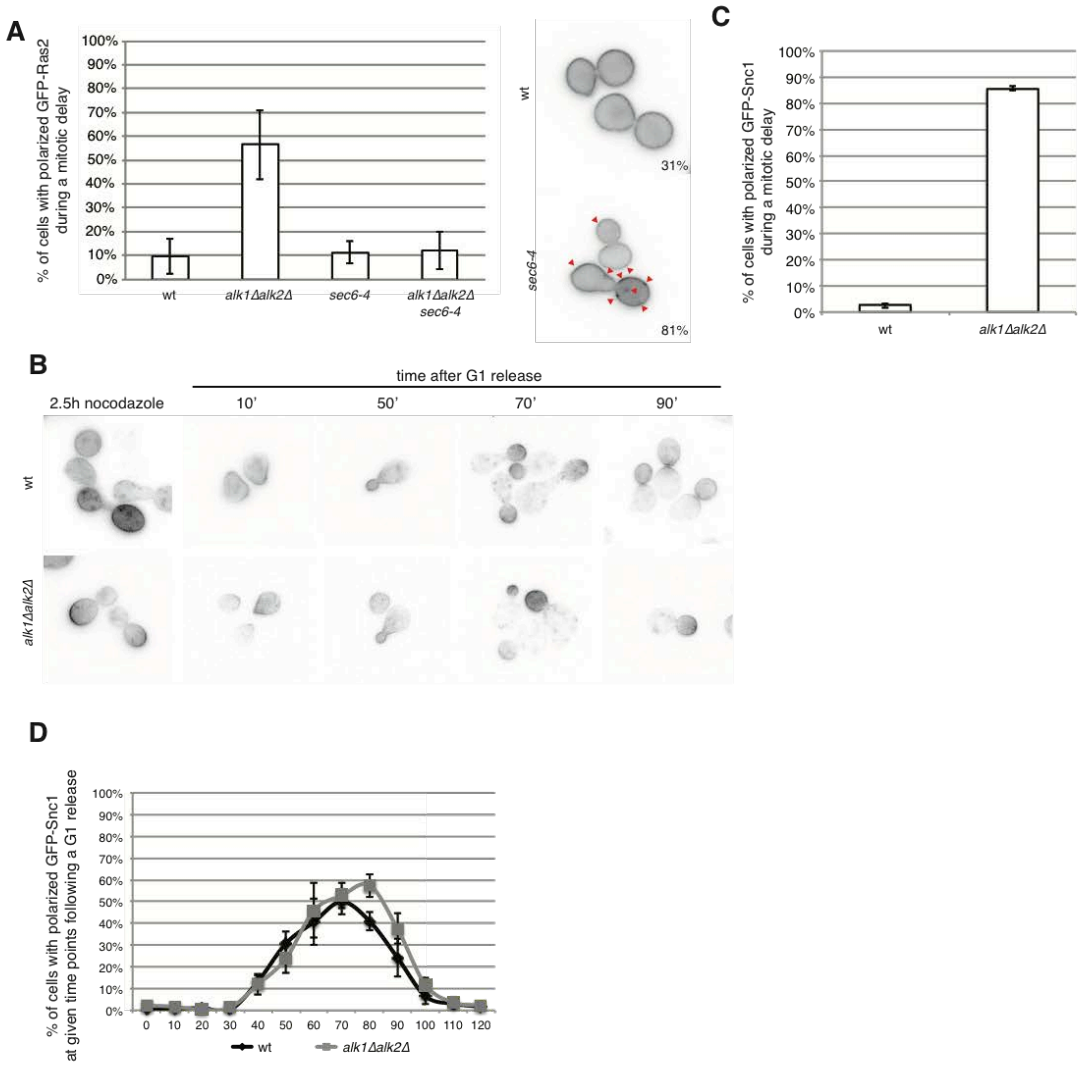
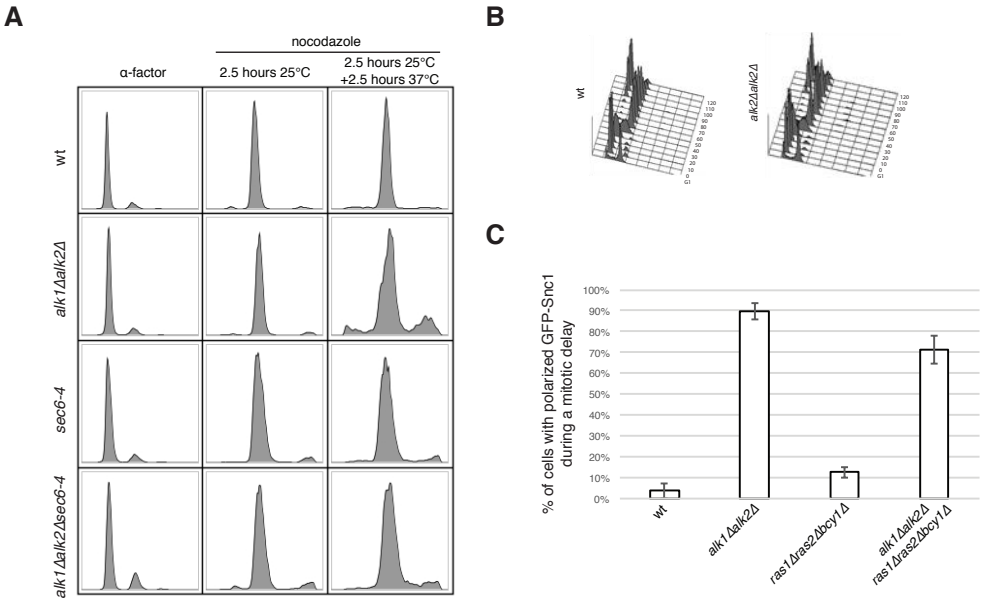


Figure 7 Haspin promotes a shift from apical- to whole PM-oriented vesicle delivery required for Ras dispersion. **a** Cells of given strains were grown at permissive temperature (25°C) in raffinose-containing medium. After G1-synchronizatoin, strains were released in nocodazole containing medium supplemented with 2% Galactose to induce expression of GFP-*RAS2*. After 2.5 hours, coltures were shifted to 37°C to inactivate exocytosis for further 2.5 hours, when samples were taken and analyzed by fluorescence microscopy. The graph represents the percentage of cells with polarized GFP-Ras2. Error bars represent standard deviation. Samples images for given strains with relative percentage of cells positive for vesicular GFP-Ras2 are shown. **b,c,d** wt or haspin-lacking cells expressing *GFP-SNC1* were arrested in G1 and then released for 2.5 hours with nocodazole (**b,c**) or released in fresh medium without drugs (**b,d**). GFP-Snc1 distribution was monitored by fluroescence microscopy at various time points; quantification of the phenotype is reported in **c** and **d**.



Supplementary Figure 7 a FACS profile for experiments described in Figure 7a,b,c. **b** Cell cycle analysis of experiment in Figure 7c,d. **c** Cells of given strains expressing GFP-Snc1 were treated with nocodazole for 3 hours. Samples were then taken to monitor distribution of the SNARE. The figure reports the percentage of cells with polarized Snc1. Error bars correspond to standard deviation.

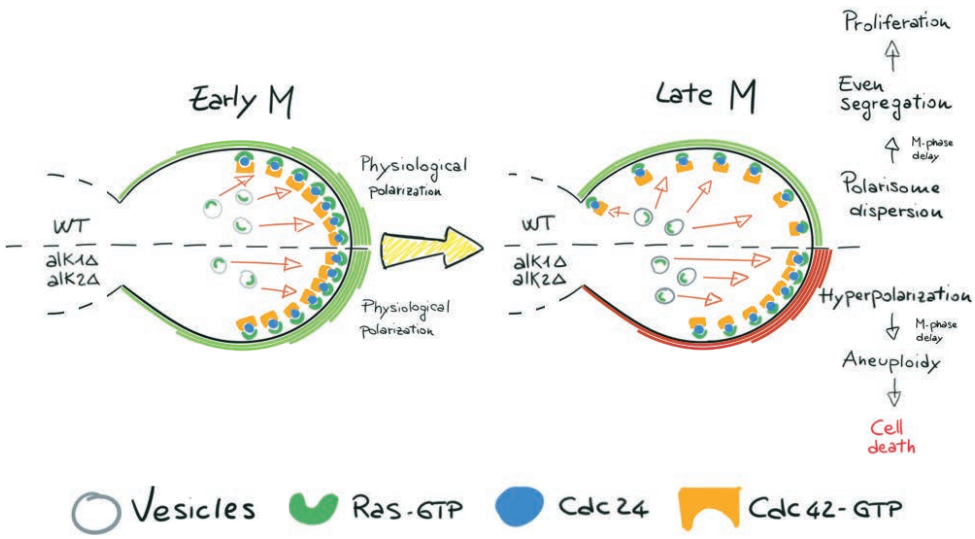
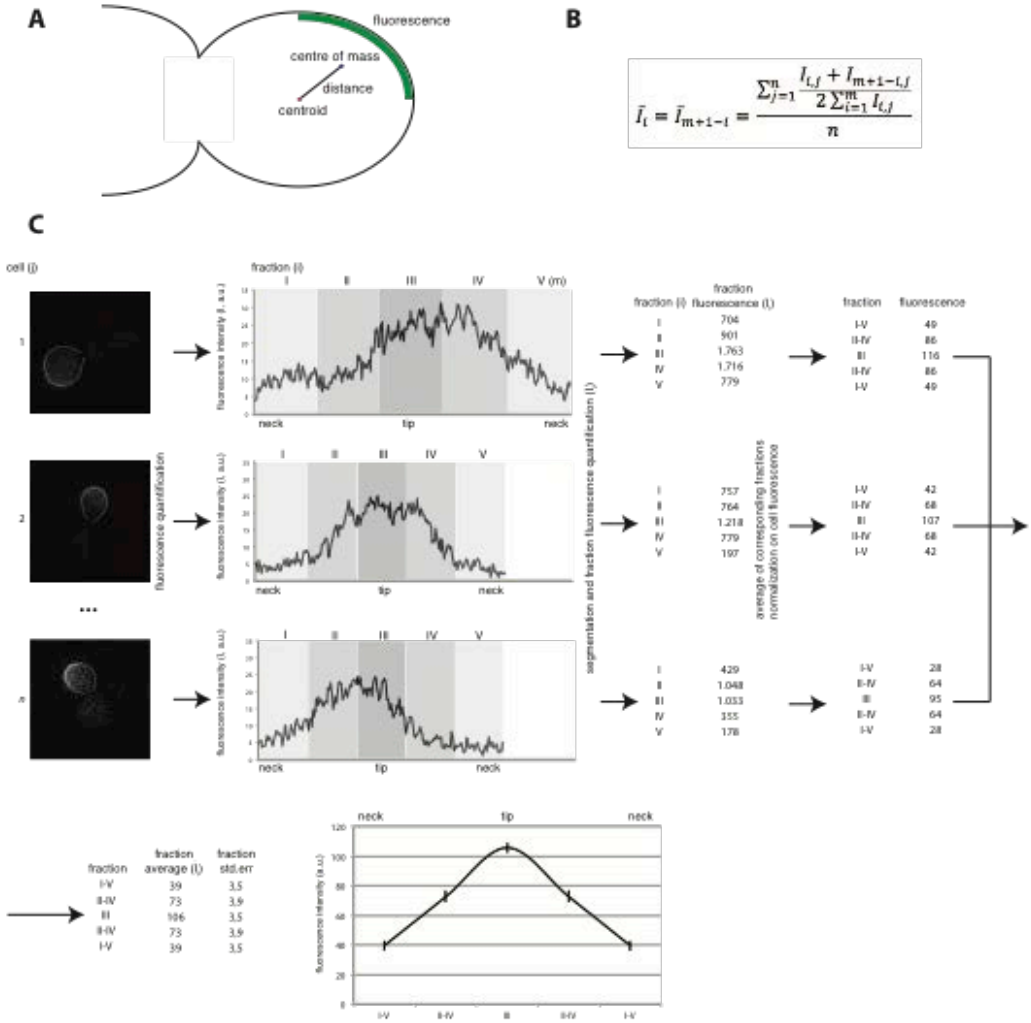


Figure 8 Haspin-dependent Ras dispersion from the bud tip is essential for a successful mitosis. During M-phase, Cdc24 relies on a physical interaction with GTP-Ras to be recruited and regulates even distribution of Cdc42 activity. In early mitosis, the exocytic vesicles that mediate Ras-GTP delivery to the PM are polarized towards the bud tip, causing accumulation of Cdc24 and hence GTP-Cdc42 to the same region. As cell cycle progresses through late mitosis, wt cells reorient their trafficking routes promoting an even vesicle delivery to the whole daughter PM, redistributing GTP-Ras, Cdc24 and active Cdc42. Loss of haspin impairs this shift in vesicle delivery, causing a persistent polarized traffic towards the bud tip with consequent persistency of the described polarity factors that, in case of mitotic delays, ultimately results in a failure in nuclear segregation and consequent cell death.



Supplementary Figure 8 Fluorescence quantifications. Figure **a** schematically represents the centroid-centre of mass distance. This value was then normalized according to cell dimension and circularity. 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.