Chapter 13

Protein Phosphorylation is an Important Tool to Change the Fate of Key Players in the Control of Cell Cycle Progression in *Saccharomyces cerevisiae*

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Additional information is available at the end of the chapter

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1. Introduction

Protein phosphorylation is a reversible posttranslational modification that can modulate protein role in several physiological processes in almost every possible way. These include modification of its intrinsic biological activity, subcellular location, half-life and binding with other proteins. Protein phosphorylation is particularly important for the regulation of key proteins involved in the control of cell cycle progression.

Protein phosphorylation is the covalent binding of a phosphate group to some critical residues of the polypeptide. The phosphorylation state of a protein is given by a balance between the activity of protein kinases and protein phosphatases. Eukaryotic protein kinases transfer phosphate groups (PO_4^{3-}) from ATP to an hydroxyl group of the lateral chain of specific serine, threonine or tyrosine residues on peptide substrates. In simple eukaryotic cells, like yeasts, Ser/Thr kinases are more common, while more complex eukaryotic cells, like human cells, have many Tyr kinases. Protein kinases recognize their substrates specifically and their active site consists of an activation loop and a catalytic loop between which substrates bind. Protein kinases differ from each other in the structure of their catalitic domain. A second class of enzymes, protein phosphatases, is responsible for the reverse reaction in which phosphate groups are removed from a protein. Phosphatases gain specificity by binding protein cofactors which facilitate binding to specific phosphoproteins. The active phosphatase often consists of a complex of the phosphatase catalytic subunit and a regulatory subunit.

The use of protein phosphorylation/dephosphorylation as a control mechanism has many advantages since it is rapid, it does not require new proteins to be made or degraded and it

is easily reversible. The phosphorylation of specific residues induces structural changes that regulate protein functions by modulating protein folding, substrate affinity, stability and activity. For example, phosphorylation can cause switch-like changes in protein function, which can also lead to major modifications in the catalytic function of enzymes, including kinases and phosphatases. In addition, protein phosphorylation often leads to rearrangement in the structure of the protein that can induce changes in interacting partners or subcellular localization.

Phosphorylation acts as a molecular switch for many regulatory events in signalling pathways that drive cell division, proliferation, differentiation and apoptosis. In order to ensure an appropriate balance of protein phosphorylation, the cell can compartmentalize both protein kinases and phosphatases. Another kind of regulation can be achieved by the spatial distribution of kinases and phosphatases, that creates a gradient of phosphorylated substrates across different subcellular compartments. This spatial separation can also control the activity of other proteins or enzymes and the occurrence of other posttranslational modifications.

Even in a simple organism like budding yeast, approximately 3% of its proteins are kinases or phosphatases. Some of these enzymes are extremely specific, indeed that are able to phosphorylate or dephosphorylate only a few target proteins, while others can act broadly on many proteins. The examples of known targets of phosphorylation include most protein components of the cell like enzymes, structural proteins, cell receptors, ion channels and signaling factors. If a protein is controlled by its phosphorylation state, its activity will be directly dependent on the activity of the kinases and phosphatases that act on it. It is quite common for a phosphate group to be added or removed from a protein continually, a cycle that allows a protein to switch rapidly from one state to another.

A protein can be modified by the addition of a single phosphate group or it can undergo multisite phosphorylation, these events can be driven by a single kinase or by multiple kinases that act in concert. Multisite phosphorylation can determine the extent and duration of a cellular response and can integrate multiple signals on the same protein. For example, many protein kinases involved in the cell cycle control function by generating phosphoSer/Thr-containing sequence motifs in their substrates that are then recognized by phosphoSer/Thr binding proteins. In several cases the phosphopeptide binding domain targets the kinase to prephosphorylated (primed) sites and then mediates processive phosphorylation of the substrate. An important example of this regulation is given by some budding yeast proteins that are phosphorylated by the catalitic subunit of the Cyclin-Dependent Kinase (CDK), Cdc28, that “primes” the protein in order to bind and to be phosphorylated by the Polo-like kinase (Plk) Cdc5.

Cdks are the master regulators of the cell cycle and Cdc5 plays key roles during all stages of mitosis and in the cell cycle checkpoint response to genotoxic stress [1,2]. The protein kinase Cdc28 drives every cell cycle transition and its activity is tightly regulated by phosphorylation/dephosphorylation events and by its association with a class of proteins called cyclins. Cyclins levels fluctuate during the cell cycle and their binding to Cdc28

activates its catalitic domain and allows its binding to specific substrates. Polo-like kinases have a conserved Ser/Thr kinase domain and a noncatalytic C-terminal region composed by two homologous boxes Polo Box Domain (PBD) [3], that appear to target the kinase to mitotic substrates. In addition, it has been shown that phosphopeptide binding to the PBD stimulates kinase activity, suggesting a conformational switching mechanism for Plk regulation and a double function for the PBD.

It is the simplicity, reversibility and flexibility of phosphorylation that explains why it has been adopted as the most general control mechanism of the cell. Below we describe how phosphorylation and dephosphorylation events can finely regulate in space and time some key proteins in the control of cell cycle progression in *Saccharomyces cerevisiae* .

2. The protein kinase Swe1 is regulated at several levels

Budding yeast cyclin-dependent kinase Cdk1 is the motor that drives cell cycle progression. Cdk1 activity is regulated at multiple levels in different cell cycle phases by interactions with different proteins, called cyclins, and by phosphorylation and dephosphorylation events. In G2 phase of the cell cycle, the protein kinase Swe1 inhibits Cdk1 by phosphorylating the conserved Y19 residue of its catalytic subunit Cdc28 [4], thus blocking both switch from polar to isotropic bud growth and nuclear division, since both these events rely on G2/M Cdk activity. This inhibitory phosphorylation is reversed by the Mih1 phosphatase [5], leading to Cdk1 activation and entry into mitosis. Swe1 phosphorylates and inhibits Cdc28 during every cell cycle and in case of problems in the bud neck, bud formation, actin cytoskeleton and abnormal cell shape. Successful bud formation leads to Swe1 degradation in late G2 phase, while morphological defects block this degradation, thus delaying entry into mitosis. Swe1 has therefore a critical role in coordinating cell morphogenesis with nuclear division.

Swe1 levels are controlled by the “morphogenesis checkpoint”, a pathway that is activated in response to alterations in the actin cytoskeleton or in septin organization. The activation of this checkpoint ultimately leads to Swe1 stabilization and a subsequent delay in nuclear division [6]. In an unperturbed cell cycle, Swe1 is recruited to the mother-bud neck in S phase; this change in its localization, which is promoted by the interaction with its regulators Hsl1 and Hsl7, is essential for subsequent Swe1 multiphosphorylation, an event that leads to its ubiquitylation and degradation, thus allowing entry into mitosis. The morphogenesis checkpoint causes Swe1 stabilization by interfering with its localization to the bud neck, acting directly on Swe1 or on its regulators Hsl1 and Hsl7, thus preventing modifications that lead to its degradation [7]. Accordingly, the lack of septin localization at the bud neck results in Swe1 stabilization [7-9], and even subtle perturbations in septin structure interfere with Hsl1 and Swe1 localization to the bud neck [7]. Moreover, actin depolymerization in budded cells causes both stabilization of Swe1 and its displacement from the bud neck without altering Hsl1 localization [7], indicating that morphogenesis checkpoint activation prevents Swe1 degradation by interfering with Swe1 localization to the bud neck, thus inhibiting its phosphorylation.

Swe1 is subjected to multiple regulations that change its phosphorylation state (35-40 phosphorylated sites have been identified *in vivo*), subcellular localization and protein levels (Figure 1). During S phase, Swe1 accumulates in the nucleus where it is phosphorylated by Clb-Cdc28 before being exported to the cytoplams and then to the daughter side of the bud neck. Swe1 nuclear export is essential for its degradation in G2/M in fact a Swe1 variant that cannot be exported from the nucleus is largely stabilized [10]. Clb-Cdc28 phosphorylates Swe1 at multiple sites [11] and these events can occur in the nucleus, in the cytoplasm or at the bud neck. This multiphosphorylation by Cdc28-Clb has different roles: on one hand it promotes Swe1 activity generating a feedback loop, while on the other hand it promotes Swe1 degradation [12]. At the mother-bud neck, filaments of conserved proteins called septins (Cdc3, Cdc10, Cdc11, Cdc12 and Shs1 in *S. cerevisiae*) form a dynamic ring structure [13] that is essential for the recruitment of a number of proteins involved in the control of cell cycle progression [14,15]. Among these, the septin ring acts as a platform to recruit several Swe1 regulators, such as the Hsl1 protein kinase and its adaptor Hsl7, both essential for Swe1 localization to the bud neck and for its phosphorylation [7,9]. Hsl1, whose kinase activity requires assembled septins, undergoes autophosphorylation [8,16] and phosphorylates Hsl7 [9] but does not seem to phosphorylates Swe1 [17] although its kinase activity is required for Swe1 recruitment at the bud neck [18]. The fact that Hsl1 activation requires assembled septins ensures that Swe1 degradation does not begin until a bud has formed, thus providing a link between bud formation and entry into mitosis [18]. Also the PAK (p21-activated kinase) kinase Cla4 associates with the septin ring and is involved in Swe1 phosphorylation, probably during S phase [19]. Moreover Swe1 phosphorylation by Clb-Cdc28 promotes subsequent phosphorylation by the Polo-like kinase Cdc5; in fact the synergistic phosphorylation that can be observed *in vitro* on Swe1 by Clb2-Cdc28 and Cdc5 is the result of priming Swe1 by Cdc28, in which the resulting phosphorylated Swe1 becomes a better substrate for Cdc5 [20]. An additional level of Swe1 regulation involves the Cdc55 regulatory subunit of protein phosphatase PP2A, that has been implicated in its degradation since loss of Cdc55 function causes Swe1 stabilization [21]. However, how this control happens at the molecular level is not clear. In addition, a mathematical model for the morphogenesis checkpoint activation suggested that a subset of Swe1 phosphorylations could inhibit its activity whereas other phosphorylations could target Swe1 for degradation [22]. In any case, hyperphosphorylated Swe1 species are recognized by a still unidentified ubiquitin ligase and ubiquitylated [23]. Subsequently, Swe1 is degraded via the proteasome and this event allows mitotic entry [23]. However, how bud neck-localized Swe1 is targeted to degradation after phosphorylation is still obscure. There are Swe1 variants that do not undergo degradation although they show proper bud neck localization, phosphorylation and interaction with known Swe1 regulators [23], indicating that still unknown Swe1 regulators exist.

So, Swe1 regulation is governed by complex pathways that are still partially unidentified. In particular, the involvement of the ubiquitylation pathway and the identity of the related ubiquitin ligase(s) involved in Swe1 degradation are unknown. A possible role in this control can be hypothesized for the functionally redundant budding yeast proteins Dma1 and Dma2, which belong to the same FHA-RING ubiquitin ligase family as *S. pombe* Dma1 and human Chfr and Rnf8. FHA domains are phosphothreonine-binding modules [24]

frequently found in DNA repair and checkpoint proteins [25,26] and RING domains are typical of E3 ubiquitin ligases [26]. The presence of an FHA domain implies that one or more protein kinases function upstream of these proteins. An intact FHA domain is required for checkpoint function of all characterized FHA-RING ubiquitin ligases. All these proteins appear to control different aspects of the mitotic cell cycle, but several molecular details of their functions are still obscure [for review, see 27]. In particular, the *S. cerevisiae* Dma proteins are involved in mitotic checkpoints and contribute to control septin ring dynamics and cytokinesis by unknown mechanisms [28,29]. Moreover, *in vitro* ubiquitin ligase activity of Dma1 and Dma2 has been described [30], although their *in vivo* targets are still unknown.

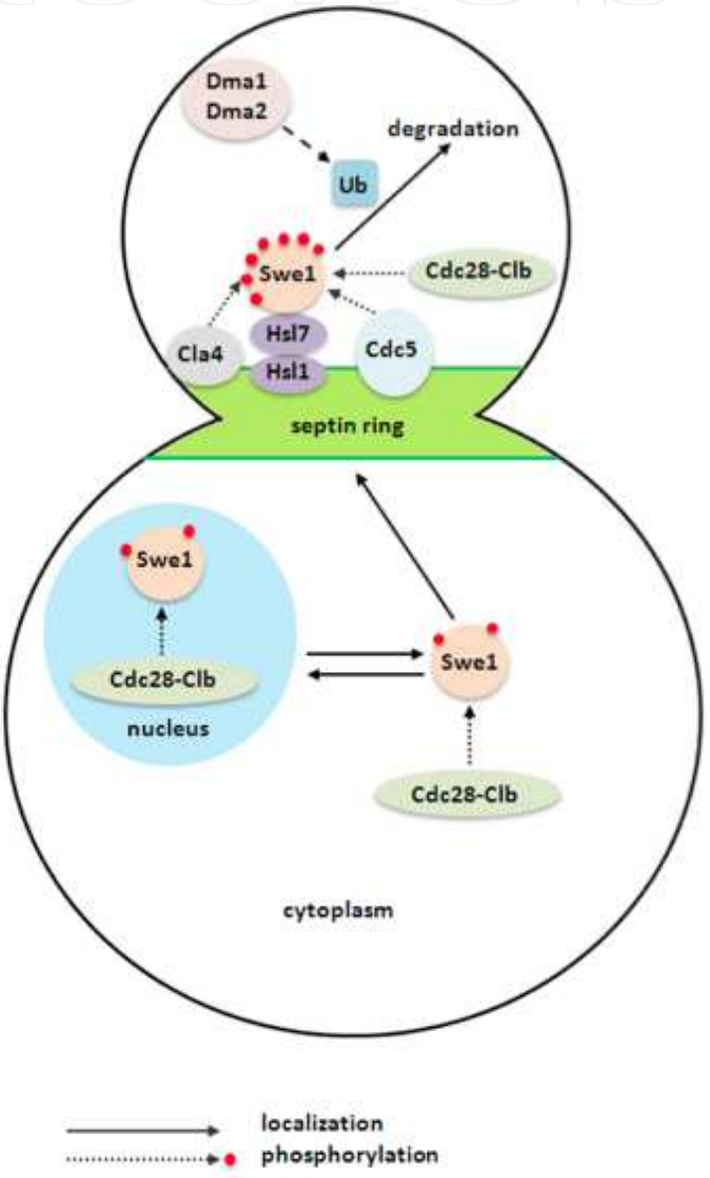

Figure 1. Model for Swe1 regulation. Swe1 shuttles from the nucleus to the cytoplasm and then it is translocated to the septin ring at the bud neck. These events are controlled through phosphorylation by Cdc28-Clb kinase. Once at the bud neck, Swe1 undergoes other phosphorylation events that drive its ubiquitylation and ultimately lead to its degradation via the proteasome. Dashed line indicates that Dma1 and Dma2 ubiquitin ligases are involved, directly or indirectly, in Swe1 ubiquitylation.

Recently, we provided genetic and biochemical evidence that Dma proteins are involved in promoting Swe1 ubiquitylation *in vivo* and contribute to the regulation of Swe1 stability by acting in a step following the recruitment of Swe1 to the bud neck and its phosphorylation [31]. Indeed, the lack of Dma proteins leads to accumulation of fully phosphorylated and bud neck localized Swe1. However, as *dma1*Δ *dma2*Δ cells are viable while Swe1 degradation is essential for cell viability, other yet unidentified ubiquitin ligases likely ubiquitylate Swe1 during an unperturbed cell cycle. The Dma-dependent Swe1 down-regulation, whose lack does not significantly affect unperturbed cell cycle progression when the other Swe1 regulatory pathways are proficient, appears to be crucial for proper response to DNA replication stress.

Collectively, the complex Swe1 regulation is an example of how phosphorylation events drive the fate of a protein. Indeed, Swe1 undergoes multiple phosphorylations that are crucial for its localization, its activity and its interaction with an E3 ubiquitin ligase that promote its degradation; moreover also some Swe1 regulators are regulated by phosphorylation, showing how this modification is largely involved in the control of cellular events.

3. The complex regulation of the protein kinase Kin4, a key player of the spindle position checkpoint

The spindle position checkpoint (SPOC) is the important pathway that blocks mitotic exit and cytokinesis in case of mitotic spindle misalignment [32]. This pathway is crucial for budding yeast since the division site is determined early in the yeast cell cycle, in late G1 concomitantly with bud site selection, while the mitotic spindle is assembled after this event. So, in order to allow proper chromosome distribution between mother and daugher cell, the mitotic spindle must be correctly aligned with respect to the mother bud axis before mitotic exit. The SPOC monitors this event and arrests mitotic progression in case of spindle misalignment. The target of the SPOC is the GTPase Tem1, that acts at the top of the Mitotic Exit Network (MEN). The MEN is a signaling cascade of protein kinases that controls both exit from mitosis and cytokinesis. At the top of the pathway, the GTPase Tem1 activates Cdc15 kinase that leads to Dbf2/Dbf20 and Mob1 activation. The MEN ultimately activates the protein phosphatase Cdc14 that leads to Cdk1 inactivation, a key event for both mitotic exit and cytokinesis [33]. The localization of all these proteins is important for their activity. The spindle pole body (SPB) component Nud1 is the platform for most MEN components localization to the spindle poles during mitosis [34]. During every cell cycle, Tem1 is kept inactive until the mitotic spindle is properly aligned respect to the mother-bud axis, thus coupling mitotic exit with nuclear division [35-37]. Tem1 regulation is complex: it is positively regulated by Lte1 [35] and it is kept inactive by the dimeric GTPase-activating protein (GAP) Bub2-Bfa1, which is the target of two protein kinases, Cdc5 and Kin4 [38-41]. Cdc5 is the budding yeast Polo-like kinase, it plays multiple functions in mitosis and cytokinesis through phosphorylation of different substrates. Cdc5 inhibits Bfa1 by phosphorylation at the anaphase onset [40,41], thus promoting timely Tem1 activation and

mitotic exit. Kin4 is a non essential serine/threonine protein kinase that plays only a minor role in mitotic progression in normal growth conditions. But, importantly, Kin4 kinase participates in the SPOC and indeed it is essential to delay cell cycle progression of cells with a misaligned spindle. In case of spindle mispositioning, Kin4 maintains Bub2-Bfa1 GAP complex active through phosphorylation of Bfa1 on residues Ser150 and Ser180 [42], these events counteract Cdc5 action on Bfa1 and thereby inhibit mitotic exit.

As already said, the subcellular localization of MEN and SPOC components is critical for their function, indeed the asymmetric distribution of MEN activators and inhibitors is one element that couples mitotic exit with correct nuclear migration. During an unperturbed cell cycle the Bub2-Bfa1 complex and Tem1 preferentially associates with the SPB that enters the daughter cell (dSPB) [35,37]. Instead the Lte1 protein, which positively regulates Tem1, is confined in the bud from the G1/S transition to telophase, when it diffuses throughout the cytoplasm of both mother cell and bud [37]. During anaphase, the SPB-associated Bub2-Bfa1 GAP complex keeps Tem1 inactive until the SPB and spindle enter the bud, where Tem1 is activated by its encounter with Lte1, thus promoting mitotic exit. Similarly to MEN factors, Kin4 binds the SPB in a Nud1 dependent manner [34,43]. During normal cell cycle progression, Kin4 localizes to the cortex of the mother cell by interaction of its C-terminal regulatory domain and this binding is a prerequisit for its loading on mother-bound SPB (mSPB) during midanaphase [44]. When spindles is correctly oriented, Kin4 and Bub2-Bfa1 are asymmetrically localized to opposite SPBs. With anaphase onset, Bub2-Bfa1 then becomes inhibited by the Cdc5 kinase thus promoting mitotic exit. On the contrary, in case of spindle misalignment, Kin4 and Bub2-Bfa1 are brought together at both SPBs [45-48]; in these conditions Kin4 prevents Bfa1 phosphorylation by Cdc5, thereby inhibiting mitotic exit and this regulation is essential for survival of cells with a misaligned spindle.

So, Kin4 subcellular localization and activity are finely regulated during an unperturbed cell cycle and in response to spindle misalignment and these events involve its phosphorylation state (Figure 2). Kin4 phosphorylation state changes during the cell cycle: it is hyperphosphorylated during late stages of mitosis but is hypophosphorylated during S-phase and early mitosis [49]. At present, the relationship between Kin4 function, phosphorylation and localization is not fully understood, but at least three regulators of Kin4 have been identified: Lte1, the protein phosphatase PP2A-Rts1 and the bud neck kinase Elm1.

Recent data indicate that Lte1 can regulate Kin4 by controlling its phosphorylation status [50]: Lte1 binds Kin4 and promotes its hyperphosphorylation and this event restricts Kin4 binding to the mSPB and prevents Kin4 that escapes the mother cell from associating with the dSPB. Importantly, this Lte1-mediated exclusion of Kin4 from the dSPB is essential for proper mitotic exit of cells with a correctly aligned spindle. Therefore, Lte1 promotes mitotic exit by inhibiting Kin4 activity at the dSPB. However, how this regulation happens remains to be determined. Indeed, Lte1 might regulate Kin4 activity itself, the Kin4 phosphatase PP2A-Rts1, the Kin4 kinase Elm1, other Kin4 kinases or the availability of Kin4 itself to be

phosphorylated. So the SPOC function could be linked to the spatial restriction of the MEN regulators Kin4 and Lte1 and inhibition of Kin4 by Lte1 [50].

On the basis of the correlation between phosphorylation status and time of Kin4 activation during the cell cycle, it was hypothesized that dephosphorylated Kin4 might be its active form and that phosphatases that promote accumulation of this form would be required for Kin4 function. Recently, the protein phosphatase PP2A and its regulatory subunit Rts1 have been identified as Kin4 regulators. In particular, the phosphatase PP2A-Rts1 is required for Kin4 dephosphorylation during cell cycle entry and to maintain Kin4 in the dephosphorylated state during S phase and mitosis [49]. In addition, PP2A-Rts1 is crucial for proper localization of Kin4 to the mother cortex and SPBs both during the cell cycle and in response to spindle position defects [49]. The phosphatase does not appear to affect Kin4 kinase activity but instead it promotes its association with SPBs, which is essential for SPOC activity. So, PP2A-Rts1 functions upstream of Kin4, regulating its phosphorylation and localization during an unperturbed cell cycle and during SPOC activation, thus defining the phosphatase as a key regulator of SPOC function [49]. However, it is still unclear how Rts1 mobilizes Kin4 at the cortex and enables its association with SPBs, indeed it might dephosphorylate Kin4 or a Kin4 interactor at SPBs.

Another key player of Kin4 regulation is the bud neck-localized Elm1 kinase [51,52]. Elm1 has been previously implicated in septin organization, bud morphogenesis, bud neck integrity and cell cycle progression [53-55]. Recently it has been shown that Elm1 contributes to the SPOC by promoting Kin4 kinase activity. Indeed, Elm1 activates Kin4 by direct phosphorylation on Thr209, a residue in the activation loop, and on additional sites at the C terminus of Kin4 [51,52]. Kin4 phosphorylation by Elm1 is critical for its kinase activity and subsequent hyperphosphorylation [49,51,52]. At present, the molecular mechanism by which phosphorylation of Thr209 influences Kin4 catalytic function is unclear. Structural studies of kinases regulated by activation loop phosphorylation indicate that the loop might function as a "sensitive switch" that controls substrate binding triggered by phosphorylation-dependent conformational changes [56]. However, the lack of Kin4 kinase activity and hyperphosphorylation observed in *elm1*Δ cells lead to reduced Kin4 binding to the mother cell cortex and SPOC deficiency. So, Elm1 function in the SPOC upstream of Kin4 by controlling its activity and localization.

Interestingly, we have recently described a new level of complexity in SPOC regulation. Indeed, we have implicated the E3 ubiquitin ligases Dma1 and Dma2 in the control of Elm1 bud neck localization [29]. Elm1 mislocalization in *dma1*Δ *dma2*Δ cells results in reduced levels of Kin4 kinase activity and asymmetry of Bub2-Bfa1 at the spindle poles of mispositioned spindles. So, Dma1, Dma2, Elm1 and Kin4 are part of the same SPOC regulatory module, with Dma proteins controlling Elm1 localization, that is in turn required for full Kin4 activation. Importantly, it is worth noting that Dma1, Dma2, Elm1 and Kin4 all are required to prevent mitotic exit in response to spindle mispositioning but not to spindle depolymerization, differently from Bub2 and Bfa1 [28,39,52].

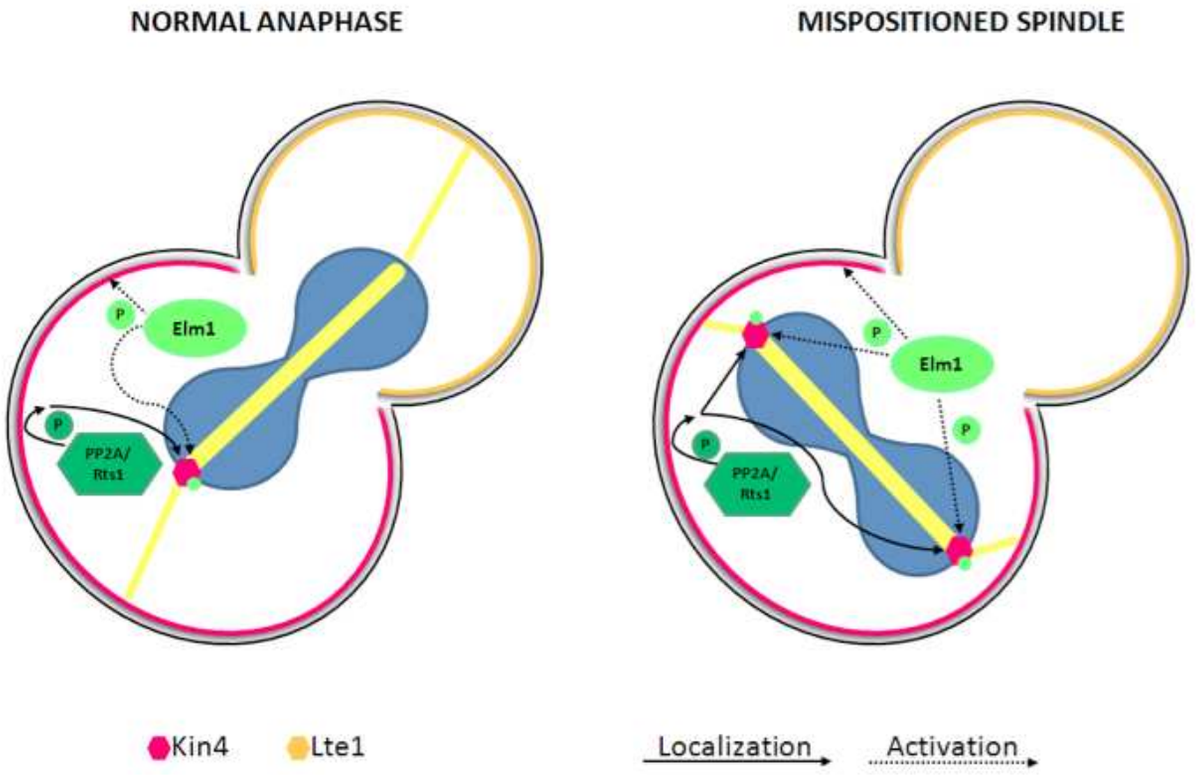

Figure 2. Model for Kin4 regulation. Kin4 is localized to the mother cell cortex and to the mother bound spindle pole body (SPB) when the mitotic spindle is correctly oriented. On the contrary, in case of spindle misalignment, Kin4 is present at both SPBs. The protein phosphatase PP2A-Rts1 is crucial for the proper localization of Kin4 to the mother cortex and SPBs both during the cell cycle and in response to spindle position defects. The protein kinase Elm1 activates Kin4 by direct phosphorylation and controls its localization.

4. Budding yeast cytokinesis is controlled by several phosphoproteins

Cytokinesis is the spatially and temporally regulated process by which, after chromosome segregation, eukaryotic cells divide their cytoplasm and membranes in order to produce two daughter cells. In budding yeast, the activity of the components of the citokinetic machinery is tightly controlled and their recruitment to the mother-bud neck follows a hieralchical order of assembly during the cell cycle. Most of these regulations are driven by phosphorylation and dephosphorylation events.

Cytokinesis completion is driven by complex and partially redundant pathways that regulate the assembly and contraction of an actomyosin ring (AMR) and the deposition of a trilaminary septum between mother and bud. In particular, the AMR is involved in constricting the plasma membrane at the division site to complete closure [57,58] and AMR contraction is coupled to the centripetal growth of the primary septum (PS).

The first step towards cytokinesis is the assembly of a septin ring, which forms at the bud neck concomitantly with bud emergence as soon as cells enter S phase, and marks the

position where constriction between mother and daughter cell will take place at the end of mitosis. During S phase, the septin ring becomes an hourglass shaped structure that serves as a scaffold for recruiting other proteins to the bud neck, among which the type II myosin heavy chain Myo1 that forms a ring at the presumptive bud site during early S phase [57]. This Myo1 ring persists at the mother-bud neck until the end of anaphase, when a coincident ring of F-actin assembles and the resulting AMR eventually contracts, accomplishing septum formation. In late anaphase Hof1, Cyk3 and Inn1 are recruited sequentially to the bud neck. All these proteins are required to activate the chitin synthase Chs2 [59-61], which is in turn recruited to the division site after mitotic exit and is required to build the primary septum (PS) which is mostly made of chitin. Once the cytokinetic apparatus is fully assembled, AMR contraction, membrane invagination and PS synthesis all begin almost immediately. After completion of the PS, at either side of this structure, secondary septa (SS), which are made of the same components as the cell wall, i. e. glucans and mannan, are synthetized [62]. At this point, mother and daughter cells are connected by a trilaminar septum. Afterwards, cell separation is driven by the action of endochitinase, Cts1, and glucanases, Dse2 Dse4 Egt2, that degrade the PS from the daughter side [63,64]. Then mother and daughter cells separate permanently from each other leaving a disk of chitin, called "bud scar", on the mother cell surface.

The events leading to cytokinesis must be tightly controlled and coordinated with chromosome segregation and mitotic exit in order to ensure the genetic stability during cell growth and thereby the fate of daughter cells. Several pathways are able to regulate the last event of the cell cycle including the Mitotic Exit Network (MEN). The MEN seems to control not only the exit from mitosis but also the timing of cytokinesis. In fact, several MEN components localize to the division site after mitotic exit and they likely play a direct role in the regulation of cytokinesis. Indeed, as mitotic Cdk1 activity decrease, MEN kinases Cdc15, Dbf2/Dbf20, Mob1, Cdc5 and the protein phosphatase Cdc14 associate with the bud neck [65-69], in addition some MEN mutants fail to undergo cytokinesis [70,71]. Here we describe examples of regulation by phosphorylation and/or dephosphorylation during cytokinesis catalyzed by Cdc28-Clb2 and/or Dbf2-Mob1 kinases and also by Cdc14 phosphatase, and how these post-translational modifications can regulate the function of three important cytokinetic proteins, Cyk3, Hof1 and Chs2, in space and time.

Cyk3 is an SH3-domain protein and was isolated as high-copy suppressor of lethality in an *iqg1Δ* strain [72] and in a *myo1Δ* strain [73]. Cyk3 interacts with Hof1 and both cooperate to recruit Inn1 [61,74], that is essential for activation of chitin synthase and therefore for primary septum formation [60,61]. Overexpression of *CYK3* leads to an actomyosin independent recruitment of Inn1 to the bud neck [74], indicating that Cyk3 plays a central role in a rescue mechanism for cytokinesis in the absence of a functional AMR. Cyk3 activity and localization are positively regulated by phosphorylation events (Figure 3). Cyk3 total levels are constant throughout the cell cycle, but interestingly Cyk3 phosphorylated species begin to accumulate after mitotic exit and this leads to its recruitment to the bud neck. This phosphorylation event requires the MEN activity [75]. In particular, it has been proposed that Cyk3 is phosphorylated by Dbf2-Mob1 kinase but if it is its direct substrate has not been determined.

It has instead been demonstrated that the Dbf2-Mob1 kinase, that appears at the division site just before AMR contraction [76,77], directly phosphorylates Hof1, a member of the F-BAR (Fes/CIP4 homology Bin/Amphiphysin/Rvsp) protein family conserved from yeast to mammals [78]. Hof1 protein levels, activity and localization are regulated by many phosphorylation events. Hof1 protein levels are regulated during the cell cycle, in particular Hof1 accumulates from G1/S phase, it disappears after mitotic exit, concomitantly with AMR contraction, and it remains unstable during the G1 phase of the following cell cycle. Hof1 colocalizes with the septin ring from S phase to late anaphase, then it interacts with the AMR before its contraction [79]. Efficient AMR contraction and cell separation are allowed by subsequent degradation of Hof1 by the action of the E3 ubiquitin ligase complex SCF (Skp, Cullin, F-box containing complex)/Grr1 [80] that can recognize its PEST (Proline, glutamin acid (E), Serine and Threonine) domain. Hof1 is directly phosphorylated by three kinases that act in concert to regulate its activity: Clb2-Cdc28, Cdc5 and Dbf2-Mob1 [81]. During mitosis, Hof1 undergoes Clb2-Cdc28 phosphorylation that does not seem to control its localization, but it primes Hof1 for subsequent phosphorylation by Cdc5 at Ser517. This event facilitates Hof1 binding to Mob1. The Dbf2-Mob1 kinase then phosphorylates several residues at both N- and C- terminal of Hof1 [81]. These modifications do not influence the physical interaction between Hof1 and Cyk3 or Inn1, instead they promote Hof1 release from the septin ring and localization to the AMR (Figure 3). There, phosphorylated Hof1 promotes AMR contraction by an unknown mechanism.

The third example is the regulation of the chitin synthase that builds the PS. Chs2 is an integral membrane protein that polymerizes chitin from its precursor UDP-N-acetyl-glucosamine. The chitin synthase 2 is synthetized in G2/M and accumulates in the endoplasmic reticulum (ER). The N-terminal and the central catalytic domain are located in the cytoplasm while the hydrophobic C-terminal domain is integrated into the ER membrane. During the metaphase to anaphase transition, Chs2 is phosphorylated in several Ser-Pro sites at its N-terminus exposed into the cytoplasm by Cdc28-Clb2 [82,83]. These phosphorylations events are important for Chs2 retention into the ER and therefore for its inibition. At the end of mitosis, MEN activation leads to the decrease of Cdc28-Clb2 activity and Chs2 can be exported from the ER and targeted to the division site [75,84-86]. The molecular mechanism of this release was unclear, but recent data indicate that Chs2 N-terminus is directly dephosphorylated by the protein phosphatase Cdc14 [86]. Then dephosphorylated Chs2 is traslocated to Golgi and, through secretory vescicoles, is delivered to the plasma membrane at the bud neck (Figure 3) [85,87]. There, Chs2 is active and promotes centripetally deposition of PS that occurs concomitantly with AMR contraction and membrane ingression. After PS completion Chs2 is removed from the bud neck by endocytosis and transferred to the vacuole where it is degraded by the action of the protease Pep4 [87]. The signal that leads to Chs2 endocytosis is currently unknown.

In summary, Cyk3, Hof1 and Chs2 regulations are good examples of how phosphorylation events can change the fate of a protein and underline the relevance of this posttranslational modification in the control of a very important cell cycle process, the cytokinesis.

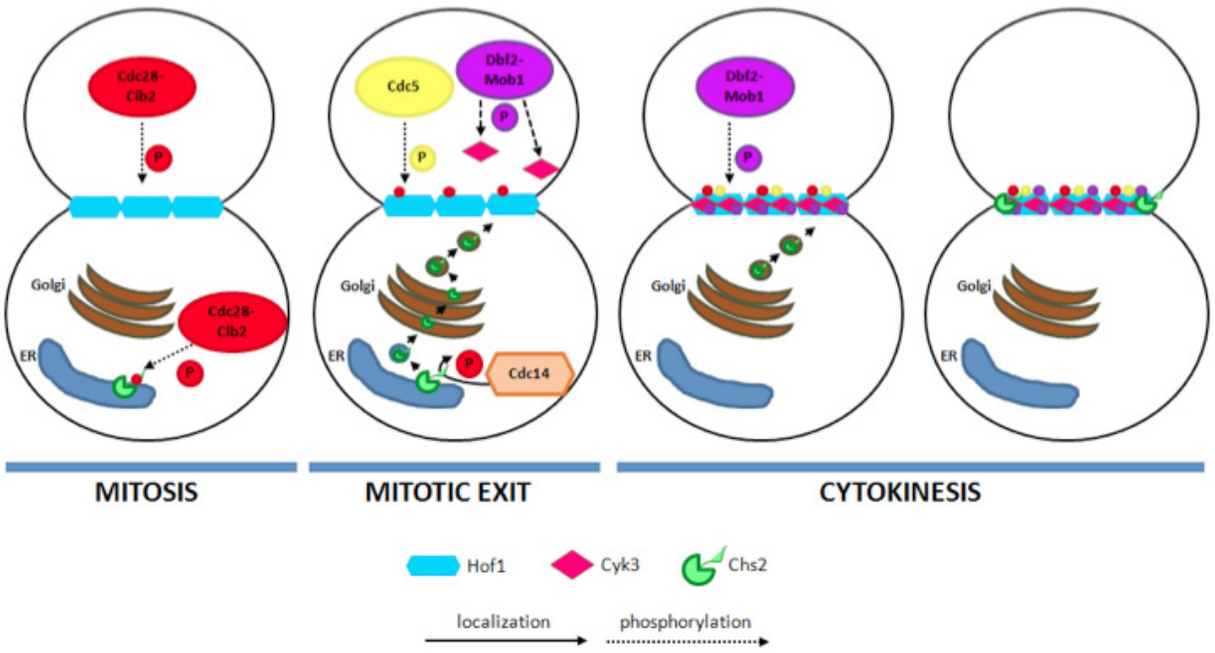

Figure 3. Model for Cyk3, Hof1 and Chs2 regulation. Cyk3 is likely phosphorylated by Dbf2-Mob1 kinase (dashed line) and recruited to the bud neck. Septin bound Hof1 is "primed" by Cdc28-Clb2 phosphorylation and then it is phosphorylated by polo-like kinase Cdc5 and by Dbf2-Mob1 kinase. These modifications promote Hof1 release from the septin ring and localization to the actomyosin ring. Chs2 accumulates in the endoplasmic reticulum (ER) with its N-terminus exposed into the cytoplasm, this tail is phosphorylated by Cdc28-Clb2. These phosphorylations events are important for Chs2 retention into the ER. At the end of mitosis, Chs2 N-terminus is directly dephosphorylated by the protein phosphatase Cdc14, then dephosphorylated Chs2 is traslocated to Golgi and, through secretory vescicoles, is delivered to the plasma membrane at the bud neck.

5. Conclusion

The phosphorylation of a protein is a simple mechanism that alters its conformation, and so its ability to function, in a reversibile way. As we can learn from the examples of protein regulation that we have focused on, phosphorylation is a flexible mechanism that regulates the target protein in several ways. Indeed, phosphorylation is not simply used to switch the activity of a protein on or off, but can have many additional roles. It can influence its ability to form complexes with other proteins, it can affect the rate at which a protein is degraded or its ability to localize to a particular subcellular location. For example, phosphorylation events on the protein kinases Swe1 and Kin4, and on the cytokinetic proteins Cyk3, Hof1 and Chs2 lead to change in their localization.

The action of kinases is counteracted by phosphatases and both controls are essential to determine the phosphorylation state of the target proteins. The balance of phosphorylation and dephosphorylation can also be critical in determining the strength and duration of the response. Therefore, kinases and phosphatases must be regulated spatiotemporally in order to obtain the proper cellular response.

In addition, a protein can be modified by the addition of a single phosphate group or by multiple phosphates, by a single protein kinase or by multiple kinases. Multisite phosphorylation is a strategy that enables two or more effects to operate in the same protein. Indeed, some phosphorylation events "prime" the protein in order to be phosphorylated by another kinase that acts subsequently in the same cellular compartment or in another location. The protein kinase Swe1 is a very good example of this kind of regulation, in fact several kinases act sequentially leading to the accumulation of hyperphosphorylated bud neck localized Swe1. Also the cytokinetic proteins Hof1 and Chs2 are the target of at least two different protein kinases that change Hof1 and Chs2 activity and localization. Alternatively, a phosphorylation event can inhibit the phosphorylation of other residues on the same protein. For example, Bfa1 phosphorylation by Kin4 inhibits phosphate group addition by the polo-like kinase Cdc5 and so keeps Bfa1 active.

Another important issue is the crosstalk between phosphorylation and other posttranslational modifications. Crosstalks can be positive or negative, thus promoting or inhibiting the subsequent modification. About phosphorylation and ubiquitylation, an increasing number of phosphoproteins are then ubiquitylated. Both Hof1 and Swe1 are hyperphosphorylated and afterwards ubiquitylated, subsequently they are targeted to degradation via the proteasome. A very interesting issue is how the specificity is determined. Indeed, phosphorylated residues of serine, threonine and tyrosine recognition by phosphobinding domains depends on the sequence of amino acids immediately around the phosphorylated residue, whereas recognition of monoUb by ubiquitin binding domains does not seem to be influenced by the primary sequence bearing the ubiquitinated lysine.

Even if there are several open questions regarding the molecular mechanisms that control the phosphoproteins that we have described, they are good examples of how the phosphorylation event can change the fate of a protein.

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