A new cell cycle checkpoint that senses plasma membrane/cell wall damage in budding yeast

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In nature, cells face a variety of stresses that cause physical damage to the plasma membrane and cell wall. It is well established that evolutionarily conserved cell cycle checkpoints monitor various cellular perturbations, including DNA damage and spindle misalignment. However, the ability of these cell cycle checkpoints to sense a damaged plasma membrane/cell wall is poorly understood. To the best of our knowledge, our recent paper described the first example of such a checkpoint, using budding yeast as a model. In this review, we will discuss this important question as well as provide hypothetical explanations to be tested in the future.

Keywords:
Cdc6; cell cycle checkpoint; plasma membrane/cell wall damage; stress response; yeast

Introduction

A cell divides into two daughter cells after an orderly sequence of replication events, known as the cell cycle. The cell cycle is composed of four phases, G1, S, G2, and M, whose transitions are triggered by the activation of key cell cycle regulators, cyclin-dependent kinase (Cdk) [1]. Cdk is cyclically activated by the binding of their activator proteins, cyclins, that are expressed at specific stages of the cell cycle. Cyclin/Cdk complexes phosphorylate and activate numerous downstream signaling cascades that promote phase-specific biological processes [2]. To accomplish faithful cell division, cells need to ensure that internal and external conditions are favorable for cell cycle progression, which is the function of cell cycle checkpoints [3]. Once a deleterious condition, such as a defect in DNA replication/damage or spindle assembly/positioning, is detected by a checkpoint, a cell transiently arrests cell cycle progression [4, 5]. Upon resolution of the problem, the cell then re-enters the cell cycle.

A common type of perturbation to the cell is plasma membrane and cell wall damage (hereafter referred to as "plasma membrane damage"). Local wounding and repair of the cell surface occurs frequently in nature. Accumulating evidence indicates that the mechanisms of local plasma membrane repair are evolutionarily conserved from yeasts to humans [6]. Plasma membrane damage is caused by various triggers, ranging from physical attacks and pathogen invasion, to physiological cellular activities such as muscle contraction [7, 8]. Despite extensive efforts to reveal the mechanisms of plasma membrane repair, it remains poorly understood how plasma membrane damage and repair are integrated with the cell cycle.

We recently found that budding yeast, an excellent model for eukaryotic cells, has a cell cycle checkpoint that monitors plasma membrane damage, which leads to transient cell cycle arrest in G1 [9]. In this review, we discuss how plasma membrane damage is sensed and how the signal is transduced to the cell cycle machinery. We also consider possible molecular mechanisms to be tested in the future.

Conserved mechanisms regulate plasma membrane repair

In higher eukaryotes, plasma membrane damage is quickly halted by the fusion of vesicles and organelles near the wound [7, 8, 10, 11]. Accumulating evidence indicates that there are at least two major mechanisms of plasma membrane repair: (i) Ca^{2+} influx from the

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Abbreviations:
APC, anaphase-promoting complex; **CDK,** cyclin-dependent kinase; **CWI,** cell wall integrity; **GEF,** guanine-nucleotide exchange factor; **GSK-3,** glycogen synthase kinase-3; **MAPK,** mitogen activated protein kinase; **MBF,** MluI-binding factor; **SBF,** SCB-binding factor; **SCF,** skp cullin F-box.

extracellular environment that promotes the extensive fusion of intracellular vesicles and lysosomes to the damaged plasma membrane [12–14] and (ii) large-scale reorganization of the cytoskeleton around the damaged plasma membrane [15]. Given that the components of these mechanisms are evolutionarily conserved from unicellular eukaryotes to humans, it is reasonable to hypothesize that the mechanism themselves could also be conserved.

A unicellular eukaryote such as budding yeast also utilizes Ca^{2+} signaling and cytoskeletal rearrangement in response to plasma membrane damage. Analogous to higher eukaryotes, large-scale cytoskeletal reorganization is controlled by Rho-type GTPase-dependent signaling [16]. In budding yeast, this pathway is called the cell wall integrity (CWI) pathway [16] (Fig. 1). The CWI pathway is composed of the cell surface sensing Wsc proteins; a Rho-type GTPase Rho1 [17, 18] and its guanine-nucleotide exchange factor (GEF) Rom1/2 [18, 19]; the protein kinase C Pkc1 [20]; and the down-stream mitogen activated protein kinase (MAPK) cascade [21]. The best-studied output of the CWI pathway is the transcriptional activation of genes that promotes cell wall synthesis [16]. In parallel, the Ca^{2+} signaling pathway likely plays key roles after plasma membrane damage because the CWI pathway and the Ca^{2+}/calmodulin-dependent calcineurin pathway show synthetic lethality [22]. Furthermore, we found that laser-induced local plasma membrane damage promoted the nuclear import of Crz1, which is the downstream transcription factor of the Ca^{2+}/calmodulin-dependent calcineurin pathway (Kono, unpublished data; Fig. 1). Taken together, these results indicate that two essential signaling pathways of plasma membrane damage are evolutionarily conserved from yeasts to humans.

The plasma membrane damage checkpoint arrests the cell cycle in late G1

Various stresses (e.g. heat shock, oxidative stress, and osmotic stress) may damage the plasma membrane and cell wall, and result in the transient growth arrest [23]. However, the detailed molecular mechanism linking the plasma membrane perturbation to the cell cycle machinery was unknown. Our recent studies revealed that plasma membrane damage transiently arrests cell cycle progression in late G1 [9], suggesting that a cell cycle checkpoint is involved in this arrest. In budding yeast, there are three G1 cyclins (Cln1-3), two S cyclins (Clb5 and Clb6), four mitotic cyclins (Clb1-4), and a single Cdk (Cdk1alias Cdc28) [24]. An in vitro kinase assay revealed that Clb5/Cdk1, a cyclin/Cdk complex required for DNA replication during S phase, had greatly reduced activity after plasma membrane damage [9]. Clb5 protein levels remained constant, which excludes the possibility that Clb5 protein is degraded and inhibited [9].

We sought to determine how Cdk is inhibited upon plasma membrane damage. In budding yeast, there are at least three major mechanisms that inhibit the S cyclin/Cdk1 complex: (i) transcriptional repression of S cyclins; (ii) protein degradation of S cyclins; and (iii) binding of the Cdk1 inhibitors. The first two possibilities were less attractive, because Clb5 is stabilized after plasma membrane damage [9]. Therefore, we examined the involvement of Sic1, a Cdk1 inhibitor. Sic1 is stabilized after plasma membrane damage [9], suggesting that inactivation of S cyclin/Cdk1 upon plasma membrane damage via Sic1 stabilization may play a role in G1 arrest (Fig. 2). However, deletion of *SIC1* failed to disrupt the plasma membrane damage checkpoint [9]. One possible explanation is that Sic1 stabilization is a consequence rather than a cause of cell cycle arrest. Alternatively, multiple layers of regulation might exist to inactivate S cyclin/Cdk1 in parallel with Sic1 stabilization.

These results raise the question as to the molecular basis of Sic1 stabilization upon plasma membrane damage. Sic1 protein levels are regulated mainly by G1 cyclin/Cdk1-dependent phosphorylation [25, 26]. Therefore, it is likely that G1 cyclin/Cdk1 complexes are inactivated after plasma membrane damage. It is of interest to examine whether G1 cyclin is regulated at transcriptional/translational and/or protein level. Another potential regulator is the Hog1 osmostress-responsive MAPK pathway. After osmotic stress, Hog1 mediates cell-cycle arrest in G1 phase via Sic1 phosphorylation in addition to the

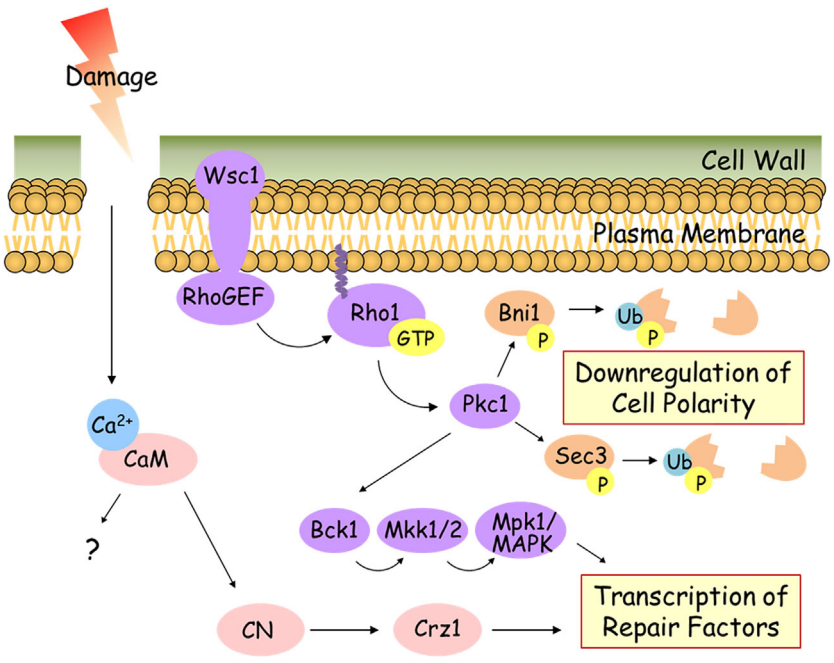

Figure 1. Plasma membrane damage activates the CWI pathway and the Ca^{2+}-dependent signaling cascade in budding yeast. Purple circles indicate the components of the CWI pathway. Pink circles indicate the components of the Ca^{2+}-dependent pathways.

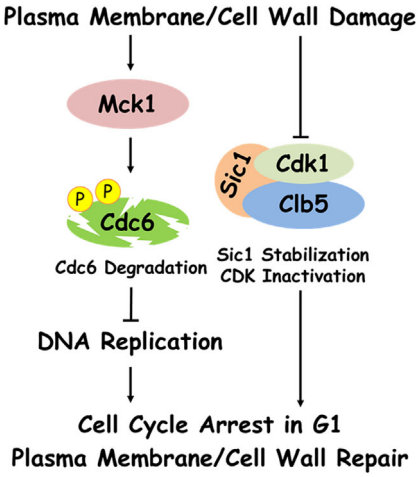

Figure 2. A current model of plasma membrane damage checkpoint in budding yeast. Plasma membrane damage promotes degradation of Cdc6 via Mck1. In parallel, the plasma membrane damage inactivates the S cyclin/Cdk complex (Clb5/Cdk1). The degradation of Cdc6 is sufficient for the inhibition of DNA replication. Yellow circles indicate phosphorylation.

downregulation of cyclins [27]. Thus, involvement of the Hog1 pathway, as well as cross talk with other MAPKs (e.g. Mpk1 and Fus3), should be examined, for example, using the phosphorylation of Sic1 as a readout.

The G1 checkpoint senses various perturbations in budding yeast

In addition to plasma membrane damage, multiple conditions are monitored by the G1 checkpoint. Yeast cells commit to the initiation of DNA replication, moving from G1 into S phase, by sensing pheromones, nutrition and cell size. This G1 control called "START" and was initially studied in the mating process [1]. A MAPK cascade inhibits G1 cyclins through activation of a Cdk inhibitor Far1, which is mediated by Fus3, the downstream kinase of the MAPK cascade [28]. As a consequence of G1 cyclin inhibition, the cell cycle is arrested at pre-START [29, 30]. Conversely, G1 cyclins phosphorylate and promote Far1 degradation, creating a positive feedback loop that results in persistent G1 arrest. To address whether a similar mechanism is used for the plasma membrane damage checkpoint, transcriptional regulation of G1 cyclins and Far1 involvement should be examined after plasma membrane damage, which can be experimentally induced by a laser or plasma membrane-damaging agents [31, 32].

Another factor monitored by the START checkpoint is cell size. Cellular concentration of Cln3, a G1 cyclin that activates the G1/S transcription factors SCB-binding factor (SBF) and MluI-binding factor (MBF), is correlated with cell size [33, 34] (Fig. 3). Recent studies have focused on Whi5 function in cell size control [35, 36]. Cln3 inhibits Whi5 via phosphorylation which promotes Whi5 translocation from the nucleus to the cytoplasm. Nuclear Whi5 inhibits SBF/MBF; therefore, Cln3-mediated Whi5 translocation allows transcriptional activation via SBF/MBF (Fig. 3). It has been proposed that the Whi5 protein amount in a cell is constant independently of cell size. Therefore, the cellular concentration of Whi5 would be lower in a large cell and higher in a small cell. This relationship would allow a cell to estimate its size by monitoring the Whi5 concentration. Thus, the Whi5-dilution model links Whi5 concentration to the cell size [35, 36]. This new scheme, termed the Whi5-dilution model, raises the possibility that Whi5 may be involved in the plasma membrane damage checkpoint. If plasma membrane damage represses G1 cyclin transcription, Whi5 could be essential for it, because Whi5 binds to and inactivates SBF/MBF, which is required for G1 cyclin transcription [37] (Fig. 3). This possibility should be investigated by testing whether the plasma membrane damage checkpoint is intact in *whi5*Δ cells.

Cdk activity underlies cell polarity competition after plasma membrane damage

We found that cell polarity competes between the tip of the daughter cell (the growth point) and the plasma membrane damage site [31]. New plasma membrane/cell wall addition occurs only at the growth point, where the bud emerges, from the late G1 to G2 phase [38]. To initiate membrane patching and cell wall synthesis at the damage site during these cell cycle stages, cell polarization toward the growth point is terminated and reestablished toward the damage site. We previously revealed that Pkc1-dependent protein degradation of key cell polarity regulators is the underlying mechanism for this polarity reorganization. Upon plasma membrane damage, Pkc1 promotes the degradation of at least two of the major cell polarity regulators at the growth point, the formin Bni1 (a linear actin regulator) and an exocyst component Sec3 (a secretion regulator) [31] (Fig. 1). Degradation of these two proteins leads to the disassembly of cell polarity complexes, causing the arrest of polarized cell growth. When Bni1 and Sec3 cannot be degraded (e.g. by expression of stabilized mutants), cells fail to initiate repair responses, resulting in cell rupture [31]. This observation indicates that downregulating cell polarization toward the growth point is a pre-requisite for the initiation of the wound healing response in cells undergoing bud formation.

Cell polarization toward the daughter cell is under the control of G1 cyclin/Cdk1 and Rho-type GTPases [38–41]. These proteins are critical for polarized actin organization and secretion, and are activated by Cdk1-dependent phosphorylation of GEFs, the Rho activators. Thus, G1 cyclin/Cdk1 needs to be inhibited to fully shut-down polarized cell growth (Fig. 4).

In contrast to the plasma membrane damage that occurs during polarized growth, the wound healing response starts immediately after laser-induced damage in cells with an actomyosin contractile ring that are undergoing cytokinesis (Kono, unpublished data). As mentioned above, cell polarization toward the growth point requires Cdk1 activity from late G1 phase to G2 phase [38]. By contrast, the formation of the actomyosin contractile ring occurs concomitant with cyclin B/Cdk inactivation [39]. These observations further support our hypothesis that inactivation of Cdk is a pre-requisite for the initiation of wound repair responses. Testing this idea by constitutive activation of Cdk1 after plasma membrane damage is an

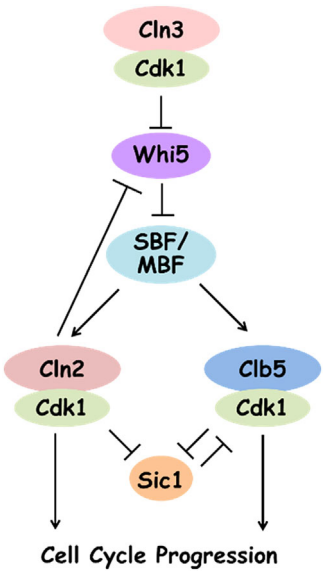

Figure 3. START control in budding yeast. Whi5 is the inhibitor of SBF/MBF, which is the central transcription factor required for the cell cycle progression in G1 and S phase. Whi5 is phosphorylated and inactivated by G1 cyclins/Cdk1. There is a feedback loop including Whi5-SBF/MBF-Cln2.

important future direction. If our hypothesis is correct, active Cdk1 will prevent wound healing processes, leading to cell lysis.

Plasma membrane damage inhibits DNA replication

The budding yeast glycogen synthase kinase-3 (GSK-3) Mck1 is a dual-specificity serine/threonine protein kinase involved in various cellular processes such as chromosome segregation, mitotic entry, and genome stability [42, 43]. Mck1 is also involved in the stress response, including temperature changes [44], microtubule stabilization by benomyl [44], and osmotic stress [45]. Furthermore, Mck1 has a role in calcineurin signaling [46–48]. Although these results suggest that Mck1 plays a key role in general stress responses, its specific role in stress-sensing and signal transduction is not well understood. We previously reported that Mck1 inhibits DNA replication after origin firing in an unperturbed cell cycle as well as after DNA damage [49]. This regulation is mediated by DNA replication factor, Cdc6 [49]. Cdc6 is an essential ATP-binding protein and a component of the pre-replicative complex (pre-RC). The pre-RC containing Cdc6, Cdt1, and Mcm2-7 proteins must be recruited to the Origin Recognition Complex (Orc1-6) at replication origins during G1 to initiate DNA replication [50]. Upon origin firing, the pre-RC has to be inhibited so that cells ensure DNA replication only once per cell cycle. For example, Cdk1-dependent Cdc6 degradation inhibits pre-RC formation, which blocks cells entering S phase until the next cell cycle [51–53].

Mck1 directly phosphorylates Cdc6 at the Thr39 and Thr368 residues, leading to proteasomal degradation of Cdc6 [49]. This mechanism plays a role in replication inhibition during unperturbed cell cycle as well as in stress response such as DNA damage. Since Mck1 is involved in stress sensing, it raises a possibility that the Mck1-Cdc6 axis may also inhibit DNA replication after plasma membrane damage. Δ*mck1* cells and cells expressing an unphosphorylatable mutant of *CDC6* (*CDC6-T39A-T368A*) failed checkpoint activation, and leading to decreased viability in the presence of plasma membrane damage [9]. Therefore, Mck1-dependent Cdc6 degradation inhibits DNA replication upon plasma membrane damage, which helps cells to survive under the stress (Fig. 2). After plasma membrane damage is repaired, a cell may restore Cdc6 level, triggering S phase progression. This possibility should be tested in the future by examining Cdc6 level after the completion of plasma membrane repair.

Priming phosphorylation is critical for phospho-degron-mediated Cdc6 degradation

We previously demonstrated that the Cdc6-Ser372 site is phosphorylated by Clb2/Cdk1 during an unperturbed cell cycle [54]. After this priming phosphorylation at Ser372, an adjacent phosphorylation site at Thr368 is phosphorylated by Mck1. The resulting double phospho-degron at Thr368 and Ser372 creates a binding site for the E3 ubiquitin ligase Cdc4 to promote protein degradation [54]. These results suggest that Clb2/Cdk1-dependent priming phosphorylation is a key factor to determine Cdc6 degradation timing. However, plasma membrane damage inhibits Clb2/Cdk1 activity in vitro (Ikui, unpublished data), which implies that another kinase phosphorylates Cdc6 under plasma membrane stress.

The priming kinase responsible for plasma membrane stress-dependent Cdc6 degradation remains to be identified. The strongest candidate is the MAPK (Mpk1) for three main reasons. First, Mpk1 is a central kinase of the CWI pathway [16]. Second, in laser damage experiment, Mpk1 is required for the damage response, but dispensable for the downregulation of cell polarization [31]. Finally, Mpk1 targets its substrate at S-P/T-P sites, which is similar to the Cdk1 consensus site. These observations support our hypothesis that Cdc6 phosphorylation sites are targeted by Mpk1 during the stress response.

We propose a model in which Cdc6 is degraded in a cell cycle-dependent manner through a Cdk1-priming mechanism (Fig. 5, top). When cells are under stress, Cdc6 is phosphorylated by an alternate kinase at the priming site, Thr372, which generates a stress-responsive phospho-degron (Fig. 5, bottom).

The next question is how the signal from local cell surface damage is transmitted to Mck1 for an appropriate response. The most upstream cue of plasma membrane damage signaling in higher eukaryotes is Ca^{2+} signaling [6]. Therefore, a plausible mechanism could be that Mck1 activity is under the control of Ca^{2+} (Fig. 6). Previous genetic analyses revealed that Mck1 is involved in a Ca^{2+}-dependent signaling pathway [46–48]. These results suggest that Ca^{2+} is one of the key triggers to initiate Cdc6 degradation after plasma membrane damage. To test this possibility, Cdc6 level after plasma membrane damage should be examined in the presence or absence of Ca^{2+} chelator such as BAPTA and EGTA. Alternatively, Mck1 could be regulated in an Mpk1-dependent manner [47]. This possibility can be tested by examining Cdc6 level and Mck1 activity after plasma membrane damage in *mpk1*Δ cells.

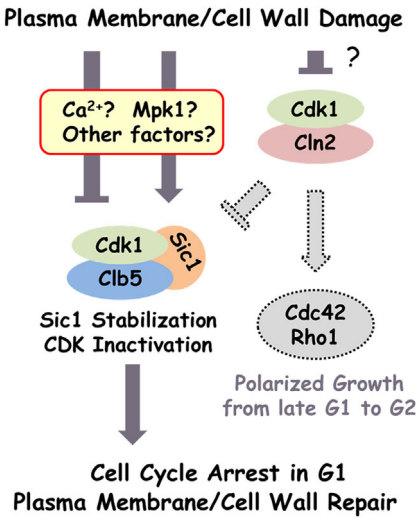

Figure 4. A model for the plasma membrane damage-dependent inhibition of polarized growth. The drawings in light gray indicate inhibited signal after plasma membrane damage.

The plasma membrane damage checkpoint in late G1 is independent of the cell wall integrity checkpoint

Yeast cells arrest the cell cycle in response to the inhibition of cell wall synthesis under the control of the cell wall integrity checkpoint [55]. The simplest model would be that failed cell wall synthesis and plasma membrane damage are monitored by the same mechanism. Unexpectedly, however, cell wall integrity checkpoint and the plasma membrane damage checkpoint are independent, because cell cycle arrest points are different between these two checkpoints. Unlike late-G1 arrest of the plasma membrane damage checkpoint, the cell wall integrity checkpoint arrests in G2/M with replicated DNA [55]. The dynactin complex (Arp1, Nip100, and Jnm1) presumably detects impaired cell wall synthesis [55], and regulates at least two downstream cascades: nuclear migration and the CWI pathway [56, 57]. The CWI pathway downregulates Hcm1 protein levels, the late-S phase transcription factor, which in turn represses *CLB2* expression, a mitotic cyclin, leading to cell cycle arrest in G2/M [58]. Arp1, a component of the dynactin complex, is dispensable for the plasma membrane damage checkpoint (Kono, unpublished data), which further supports our conclusion that these two checkpoints are independent.

The identification of the actual upstream sensor(s) for both checkpoints remains a key issue to be addressed. The most likely candidates for both checkpoints are Wsc proteins, which are transmembrane proteins that sense mechanical force and cell surface stress and are the most upstream factors of the CWI pathway [59]. However, this idea leads to an intriguing question: how does activation of the same sensor would result in differential cell cycle arrest? Alternatively, dynactin complex itself is a sensor for impaired cell wall synthesis, and it indirectly affects CWI pathway. Detailed characterization of the signaling pathway for each checkpoint is required to address this issue in the future.

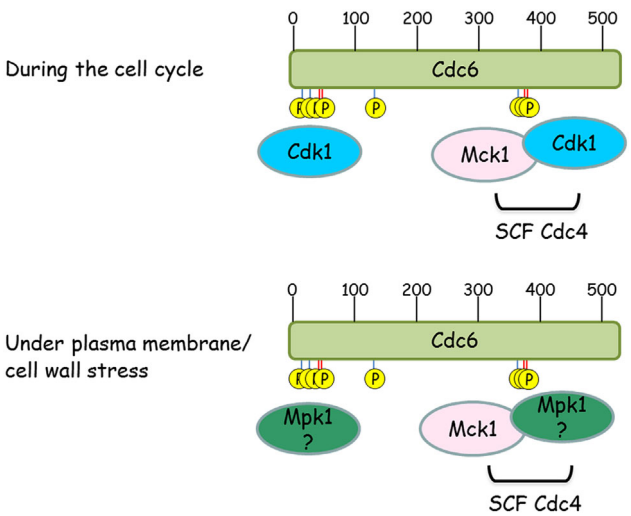

Figure 5. Stress-responsive phospho-degron in Cdc6. During the unperturbed cell cycle, Cdk1 induces the priming phosphorylation to Cdc6 for the subsequent phosphorylation by Mck1 (top). After the plasma membrane damage, an alternate priming kinase, such as Mpk1, may phosphorylate Cdc6, which allows further phosphorylation by Mck1 and protein degradation (bottom). Yellow circles indicate phosphorylation. Numbers indicate amino acids. Red lines indicate GSK-3 consensus sites. Blue lines indicate Cdk1 consensus sites.

Is the plasma membrane damage checkpoint evolutionarily conserved?

Whether plasma membrane damage is monitored by similar checkpoints in higher eukaryotes remains to be determined. All the core components of the checkpoint, including the Rho-Pkc1-MAPK cascade, GSK-3, Cdc6, the proteasome, and the cell cycle system, are evolutionarily conserved. In particular, START control is highly conserved from yeasts to humans, despite the fact that some of the key components, including Rb proteins and Cip/Kip proteins, have no obvious sequence homology to Whi5 and Sic1, respectively [60]. Thus, the plasma membrane damage checkpoint is likely to be conserved in higher eukaryotic cells. Plasma membrane damage induces cell cycle arrest in mammalian non-transformed cell lines (Kono, unpublished data). Although the signaling cascade underlying these effects should be dissected using the laser damage assay and biochemical analyses, these results open new avenues to expand this field of research.

Conclusions and outlook

In this review, we have provided an outline of recent findings and future directions regarding the plasma membrane repair mechanisms and their association with cell cycle. The central factors to be addressed are the

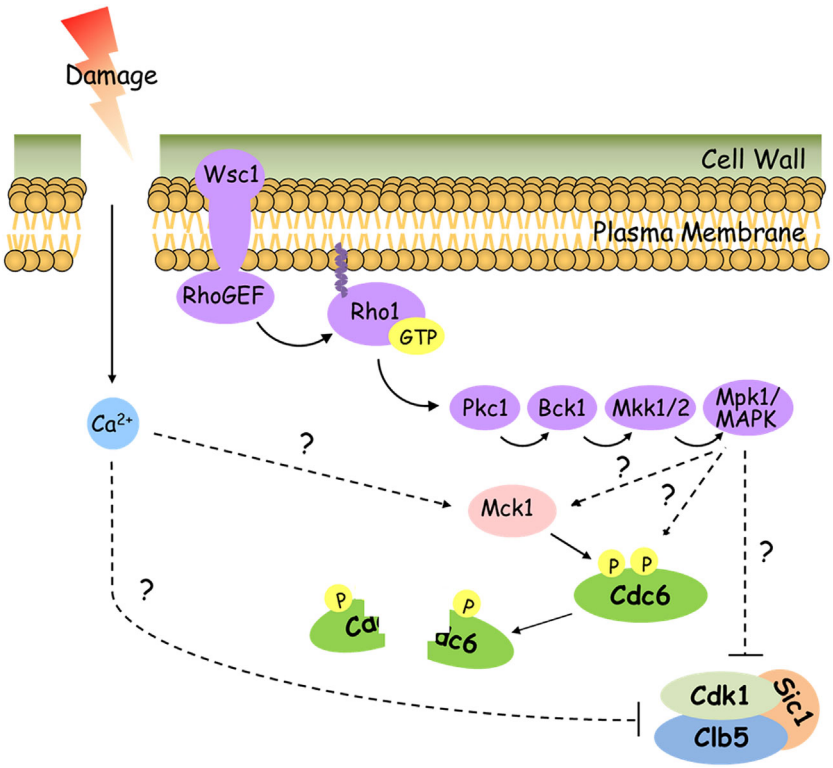

Figure 6. A working model of the plasma membrane damage checkpoint. The plasma membrane damage checkpoint activates the CWI pathway and the Ca^{2+}-dependent signaling cascade. The main question that remains to be addressed is how these two pathways are integrated to cell cycle regulations.

identification of the specific sensor and the determination of the molecular hubs linking the damage signaling and cell cycle system. The cell polarity competition after plasma membrane damage implies that Cdk1 and the damage repair pathway inhibit each other. Thus, clarifying the molecular hubs of this inhibitory loop will provide significant progress toward revealing the whole mechanism of plasma membrane repair. To identify the molecules required for this regulatory circuit, cell polarity changes after plasma membrane damage should be investigated in mutants lacking the candidate genes. The most likely candidate is the MAPK cascade, which translates gradual signal changes to all-or-none signals via positive and negative feedback. We hope to elucidate a clearer picture of this mechanism in the future.

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