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CELL SCIENCE AT A GLANCE

Greatwall kinase at a glance

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ABSTRACT

Mitosis is controlled by a subtle balance between kinase and phosphatase activities that involve the master mitotic kinase cyclin-B–Cdk1 and its antagonizing protein phosphatase 2A-B55 (PP2A-B55). Importantly, the Greatwall (Gwl; known as Mastl in mammals, Rim15 in budding yeast and Ppk18 in fission yeast) kinase pathway regulates PP2A-B55 activity by phosphorylating two proteins, cAMP-regulated phosphoprotein 19 (Arpp19) and α-endosulfine (ENSA). This phosphorylation turns these proteins into potent inhibitors of PP2A-B55, thereby promoting a correct timing and progression of mitosis. In this Cell Science at a Glance article and the accompanying poster, we discuss how Gwl is regulated in space and time, and how the Gwl–Arpp19–ENSA–PP2A-B55 pathway plays an essential role in the control of M and S phases from yeast to human. We also summarize how Gwl modulates oncogenic properties of cells and how nutrient deprivation influences Gwl activity.

KEY WORDS: Greatwall, Mastl, Cancer, Meiosis, Mitosis

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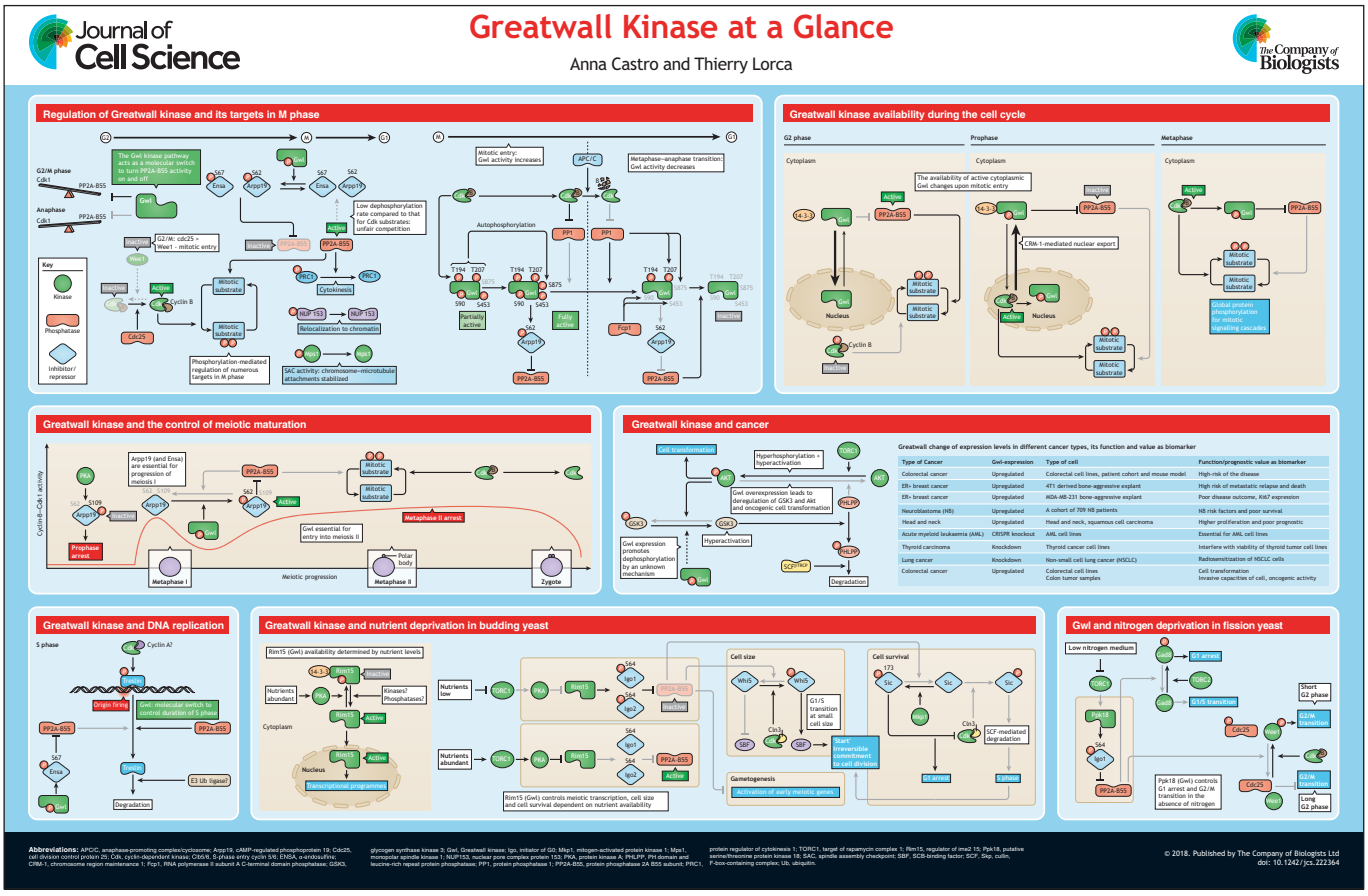
Introduction

Greatwall (Gwl, known as Mastl in mammals, Rim15 in budding yeast and Ppk18 in fission yeast) is a member of the AGC family of kinases (Blake-Hodek et al., 2012; Vigneron et al., 2011) whose main role is the inhibition of PP2A-B55 [protein phosphatase 2A (PP2A) holoenzyme containing a B55-family regulatory subunit; note there are four B55 isoforms, α–δ, encoded by *PPP2R2A*, *PPP2R2B*, *PPP2R2C* and *PPP2R2D*, respectively], the phosphatase that counterbalances cyclin-B–Cdk1 activity and that is responsible for the dephosphorylation of mitotic substrates (Alvarez-Fernandez et al., 2013; Burgess et al., 2010; Castilho et al., 2009; Cundell et al., 2013; Vigneron et al., 2009). By phosphorylating Arpp19 and ENSA, its sole substrates identified



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so far, Gwl promotes binding to and the inhibition of PP2A-B55. It thereby ensures proper protein phosphorylation timing and mitotic entry and progression (Gharbi-Ayachi et al., 2010; Mochida et al., 2010). The Gwl–Arpp19–ENSA–PP2A-B55 signalling pathway is highly conserved from yeast to humans and has been investigated in different model organisms. It is established that loss of Gwl in cells results in mitotic abnormalities that include chromosome misalignment, or mis-segregation and cytokinesis defects owing to the reactivation of PP2A-B55 and to massive mitotic substrate dephosphorylation (Alvarez-Fernandez et al., 2013; Burgess et al., 2010; Voets and Wolthuis, 2010). Although not fully elucidated, the identity of the mitotic regulators whose dephosphorylation contributes to these mitotic phenotypes has begun to be discovered. Here, we provide a broad overview of the current knowledge on the mechanisms of action of Gwl kinase through PP2A-B55 inhibition, its regulation and its main physiological functions.

The Gwl–Arpp19–ENSA–PP2A-B55 axis

Entry into mitosis is governed by the activation of cyclin-B–Cdk1. During the G2 phase, this complex is kept in its inactive state by inhibitory phosphorylations on Cdk1. These phosphorylations are controlled by the balance between Wee1 kinase and Cdc25 phosphatase (see poster). At the G2/M transition, the balance shifts from activated Wee1 to activated Cdc25, thereby resulting in the dephosphorylation of the inhibitory residue, cyclin-B–Cdk1 activation and mitotic entry. However, mitosis is also dependent on the inhibition of the protein phosphatase PP2A-B55, the antagonist of cyclin-B–Cdk1-dependent phosphorylation, through the Gwl signalling cascade. Initial studies that defined Gwl as an essential mitotic regulator were performed in *Drosophila* and *Xenopus* egg extracts (Yu et al., 2004; Vigneron et al., 2009). Accordingly, fruit flies with mutations in Gwl display undercondensed mitotic chromosomes and delayed nuclear envelope breakdown (NEB) and anaphase progression (Yu et al., 2004). Moreover, Gwl depletion in *Xenopus* egg extracts promotes mitotic exit through massive protein dephosphorylation, even in the presence of fully active cyclin-B–Cdk1 (Vigneron et al., 2009). These data pointed to a regulatory role of Gwl in the control of a phosphatase that counterbalances cyclin-B–Cdk1 activity; such a phosphatase was subsequently identified as being PP2A-B55 (Castilho et al., 2009; Mochida et al., 2009; Vigneron et al., 2009) (see poster). The mechanisms by which Gwl inhibits PP2A-B55 were then revealed in two key studies through the identification of two unique substrates of Gwl: the small and unstructured proteins Arpp19 and ENSA (Gharbi-Ayachi et al., 2010; Mochida et al., 2010). These proteins are highly similar and their phosphorylation by Gwl at a serine residue located in the highly conserved ‘FDSpGDY’ motif promotes their binding to PP2A-B55 and inhibition of the phosphatase (Gharbi-Ayachi et al., 2010; Mochida et al., 2010). Interestingly, ENSA and Arpp19 are themselves PP2A-B55 substrates; however, although they display a high affinity towards this phosphatase, their dephosphorylation rate is orders of magnitude lower than Cdk1-phosphorylated substrates. Consequently, these two proteins compete with Cdk1-phosphorylated substrates and their dephosphorylation is blocked by ‘unfair competition’ (Williams et al., 2014) (see poster).

Studies in mouse and human cells corroborated the above findings. Knockdown of the Gwl homologue microtubule-associated serine/threonine-protein kinase-like (Mastl) in HeLa cells promoted substantial mitotic phenotypes, including the presence of undercondensed DNA, chromosome misalignment and mis-segregation and severe cytokinesis defects (Burgess et al., 2010; Voets and Wolthuis, 2010). These phenotypes were caused by the improper dephosphorylation of cyclin-B–Cdk1 substrates by reactivated PP2A-B55, since they were fully rescued by the chemical inhibition of the phosphatase (Alvarez-Fernandez et al., 2013; Burgess et al., 2010; Voets and Wolthuis, 2010). The analysis of Mastl-knockout phenotypes allowed the characterization of indirect (APC3, also known as CDC27) and direct [monopolar spindle kinase 1 (Mps1; also known as TTK), protein regulator of cytokinesis 1 (PRC1) and nuclear pore protein 153 (NUP153)], PP2A-B55 substrates. The indirect APC3 phosphorylation that is observed in Mastl-depleted prometaphase cells was associated with a reduced binding of cyclin B1 to the anaphase-promoting complex (APC). This decreased interaction specifically stabilizes cyclin B without affecting other APC substrates, such as geminin or securing, and resulted in impaired sister chromatid segregation and DNA bridge formation, likely through cyclin-B–Cdk1-dependent inhibition of separase (Voets and Wolthuis, 2015).

The spindle assembly checkpoint (SAC) protein Mps1 is directly dephosphorylated by PP2A-B55 at the metaphase–anaphase transition. Mps1 phosphorylation takes place at the kinetochores, where it is essential for the recruitment of a number of other SAC proteins, such as mitotic arrest deficient gene 1 (Mad1; MAD1L1 in mammals) (Diril et al., 2016). Phosphorylation and activity of Mps1 is reduced in Mastl-knockout mouse embryonic fibroblasts (MEFs) during late prometaphase and results in a decreased kinetochore localization of the essential SAC protein Mad1, a phenotype that is not observed upon chemical inhibition of PP2A by okadaic acid (OA) (Diril et al., 2016).

PRC1 has also been identified as a direct PP2A-B55 target during cytokinesis (Cundell et al., 2013). PRC1 is a component of the anaphase central spindle that is required to promote anaphase spindle elongation and cytokinesis (Li et al., 2018). During mitosis, cyclin-B–Cdk1-dependent phosphorylation of PRC1 prevents its binding to the mitotic spindle. At the metaphase–anaphase transition, upon cyclin-B–Cdk1 inactivation, PRC1 is dephosphorylated, allowing its interaction with the central spindle microtubules to promote spindle elongation and cytokinesis (Jiang et al., 1998). PP2A-B55 reactivation by means of depletion of Mastl in HeLa cells promotes precocious dephosphorylation and recruitment of PRC1 to metaphase spindles and improper cytokinesis, a phenotype that can be partially rescued by PP2A-B55 inactivation (Cundell et al., 2013). Finally, Mastl is also essential for nuclear pore reformation by promoting dephosphorylation of the NUP153 and its subsequent recruitment to chromatin. Accordingly, Mastl depletion in Hela cells results in precocious NUP153 dephosphorylation and its premature relocalization to anaphase chromatin (Cundell et al., 2016).

Collectively, these data confirm that Mastl by controlling PP2AB55 activity is essential for timely mitotic progression by regulating phosphorylation of many key substrates that are involved in anaphase entry, cytokinesis and nuclear pore reformation (see poster).

Regulation of Gwl

Gwl activity

Two different mechanisms that regulate Gwl, via its kinase activation and modulation of its localization, have been described so far. Gwl kinase activity is tightly controlled throughout the cell cycle; it is low during G1, S, and G2 phases, drastically increases at mitotic entry and decreases again at the metaphase–anaphase transition (Hara et al., 2012; Voets and Wolthuis, 2010; Yu et al., 2006). Activation of Gwl at mitotic commitment takes place in two successive steps. First, cyclin-B–Cdk1 phosphorylates Gwl at its

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kinase domain (T193 and T206 in *Xenopus*, or T194 and T207 in humans). This phosphorylation partially activates Gwl that results in a subsequent autophosphorylation at its C-terminal domain (S883 and S875, for *Xenopus* and humans respectively) and its full activation (see poster) (Blake-Hodek et al., 2012; Vigneron et al., 2011). Besides these major phosphorylations, two additional phosphorylated residues of Gwl on *Xenopus* S89 or S90 (human S465 or S453) appear to participate in Gwl activation. However, the identity of the kinase(s) responsible for of these additional phosphorylations and their exact effect on Gwl activity remain elusive (Della Monica et al., 2015). At the metaphase–anaphase transition, concomitantly with cyclin B degradation, Gwl activity is downregulated by dephosphorylation. The activation of three phosphatases – protein phosphatase 1 (PP1), PP2A-B55 and RNA polymerase II C-terminal domain phosphatase (Fcp1, also known as CTDP1) – is essential for Gwl inactivation (Della Monica et al., 2015; Heim et al., 2015; Ma et al., 2016). PP2A-B55 and PP1 are inactivated during mitosis; the former by Arpp19 and/or ENSA, and the latter through its direct phosphorylation by cyclin-B–Cdk1 (Wu et al., 2009). The reactivation of PP1 by auto-dephosphorylation on its cyclin-B–Cdk1 site (T320), which stems from cyclin B degradation, is likely the major factor that prompts Gwl inactivation (Heim et al., 2015; Ma et al., 2016; Ren et al., 2017). Upon anaphase entry, active PP1 promotes dephosphorylation of S875 and partial inactivation of Gwl, which in turn results in a partial activation of PP2A-B55. Active PP2A-B55 will then dephosphorylate Gwl on both T194 and S875 residues (human numbering) and fully inactivate this kinase (see poster) (Heim et al., 2015; Ma et al., 2016; Rogers et al., 2016). Fcp1 additionally targets dephosphorylation of Gwl on S90 and S453 (Della Monica et al., 2015). Although, as mentioned above, the exact effect of these dephosphorylation events on Gwl activity is not known, they also appear to be essential for the complete inactivation of this kinase.

Gwl localization

Gwl is predominantly nuclear during interphase and becomes cytoplasmic at prophase just prior to nuclear envelope breakdown (NEB) (Alvarez-Fernandez et al., 2013; Wang et al., 2013; Yamamoto et al., 2014). In agreement with the nuclear localization of Gwl, importin-α and -β have been found to be associated with this kinase in *Xenopus* S3 cells (Yamamoto et al., 2014). Importin binds a functional nuclear localization signal (NLS) on Gwl, and this binding is essential for the function of Gwl in promoting mitotic entry (Yamamoto et al., 2014). In *Drosophila*, two NLSs that are present in the central region of Gwl mediate the nuclear localization of this kinase during interphase. However, at mitotic entry, the phosphorylation of these NLS by cyclin-B–Cdk1 promotes its cytosolic relocalization in a manner that is dependent on exportin-1 (CRM1) (see poster) (Wang et al., 2016). Moreover, additional phosphorylation of these sequences by Polo kinase promotes Gwl binding to 14-3-3ε and its cytoplasmic retention (Wang et al., 2013). Although conserved, the mechanisms that modulate Gwl localization in MEFs appear to be partially different. Unlike for Gwl in *Drosophila*, mouse Mastl contains a unique NLS and three active nuclear export sequences (NESs), and its cytoplasmic retention depends on its kinase activity, but does not require Polo [polo-like kinase 1 (Plk1) in mammals]-dependent phosphorylation (Alvarez-Fernandez et al., 2013). Whatever the regulatory pathway, the localization of Gwl in the cytosol appears to be essential to ensure inhibition of PP2A-B55 and subsequent massive protein phosphorylation and mitotic entry. In summary, both accurate kinase activation and localization are essential for a correct timely phosphorylation of Gwl substrates.

Cellular pathways controlled by Gwl

Gwl controls meiotic maturation

Mastl-knockout mouse oocytes complete meiosis I without dramatic defects and extrude their polar bodies normally; however, they do not progress into metaphase II and arrest with reformed nuclei (Adhikari et al., 2014). This phenotype partially differs from that seen with porcine oocytes, where the knockdown of this kinase prevented metaphase I entry in 30% of the oocytes. Remaining oocytes progressed through meiosis I, but equally displayed abnormal meiotic spindles and chromosome condensation and congression defects (Li et al., 2013). Similar abnormalities in anaphase I were observed in starfish oocytes, where Gwl activation upon addition of 1-methyladenine was prevented by the injection of neutralizing antibodies against this kinase (Okumura et al., 2014). Taken together, these data suggest a minor role of Gwl in meiosis I progression, but an essential role of this kinase in meiosis II entry. Conversely, although Arpp19 and ENSA appear to be essential for proper meiotic progression in this case, most data converge to suggest that the principal function of these substrates is in metaphase I progression. Accordingly, ENSA knockdown in mouse oocytes and the absence of Ensa in *Drosophila* mutant oocytes prevents prophase I exit (Von Stetina et al., 2008; Matthews and Evans, 2014), whereas, once phosphorylated by Gwl, Arpp19 promotes entry into meiotic divisions (Dupre et al., 2013). Arpp19 is additionally phosphorylated on S109 by the protein kinase A (PKA) during prophase I in *Xenopus* oocytes. In these oocytes, the addition of progesterone promotes downregulation of PKA and triggers meiotic resumption. Interestingly, progesterone induces a partial dephosphorylation of Arpp19 on S109 followed by a Gwl-dependent phosphorylation on S62. However, S109 phosphorylation of Arpp19 by PKA might be required to maintain prophase I arrest (Dupré et al., 2014). Accordingly, *Xenopus* oocytes overexpressing a phosphomimetic Arpp19-S109D mutant fail to resume meiosis upon progesterone addition. Arpp19 could thus switch from a S109-phosphorylated form that acts as a negative regulator essential for prophase arrest to a S62-phosphorylated form required to induce meiotic progression (see poster). Taken together, these data further indicate that the Gwl–Arpp19–Ensa–PP2A-B55 axis acts as a molecular switch to turn PP2A-B55 activity on and off.

Gwl and cancer

Recent evidence from a variety of model systems has shown that, besides its primordial role in mitotic division, Gwl also contributes in a significant extent to tumorigenic processes (Alvarez-Fernandez et al., 2017; Uppada et al., 2018; Vera et al., 2015; Dahlhaus et al., 2016; Nagel et al., 2015). Upregulation of Gwl activity promotes cell transformation and increases cell proliferation in several immortalized cells lines and in primary human fibroblasts (Vera et al., 2015). Moreover, Gwl overexpression significantly increases the invasive and migrative capacities of breast and colon cell lines, whereas its knockdown results in a drop of these oncogenic properties. Although it is not fully understood how the overexpression of Gwl contributes to tumorigenic phenotypes, recent studies shed light on the underlying signalling mechanisms. Gwl overexpression induces dephosphorylation of inhibitory residues on glycogen synthase kinase 3 (GSK3) and the subsequent hyperactivation of this kinase (Vera et al., 2015). Hyperactivated GSK3 in turn phosphorylates PHLPP and promotes its subsequent ubiquitylation and degradation mediated by the Skp1-

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Cullin-F-box (SCF) complex containing βTrcP (Li et al., 2009). Since PHLPP proteins target dephosphorylation of the serine/threonine protein kinase Akt on S473 (Gao et al., 2005), its increased proteolysis finally results in the hyperphosphorylation and hyperactivation of Akt proteins, and in increased oncogenic properties (see poster). It remains to be understood how Gwl promotes GSK3 dephosphorylation and activation. In line with a role of Gwl in oncogenesis, its overexpression has been identified in a myriad of human tumours in the last three years, including breast (Alvarez-Fernandez et al., 2017; Zhuge et al., 2017), colon (Vera et al., 2015) and head and neck squamous carcinomas (Wang et al., 2014). Moreover, Gwl levels have evolved as a biomarker in these cancers with prognostic value for survival, recurrence and metastasis (Alvarez-Fernandez et al., 2017; Wang et al., 2014; Zhuge et al., 2017). Additionally, Gwl has been highlighted as a potential new therapeutic target for several cancers, such as acute myeloid leukemia (Tzelepis et al., 2016), head and neck squamous cell carcinoma (Wang et al., 2014) and thyroid carcinoma (Anania et al., 2015).

Gwl, DNA replication and the DNA damage checkpoint

Studies on the Gwl–Arpp19–ENSA pathway have been mostly restricted to mitotic divisions; however, importantly, our recent work highlights the role of this pathway in the control of S phase (Charrasse et al., 2017). Gwl–ENSA–PP2A-B55 controls S phase length by regulating replication origin firing. Accordingly, knockdown of Gwl or ENSA in mammalian cells promotes a drop in the levels of the limiting replication factor treslin that is essential for origin firing. During unperturbed S phase, Cdk-dependent phosphorylation of treslin triggers its recruitment to the replication origins where it promotes its activation (Fragkos et al., 2015). We showed that the Gwl or Ensa knockdown phenotype is caused by the PP2A-B55-dependent dephosphorylation and the subsequent cullin-dependent ubiquitylation and proteasomal degradation of treslin. However, proteolysis of treslin that is induced by depletion of Ensa is rescued by PP2A-B55 inhibition, thereby preventing an extension of S phase (see poster) (Charrasse et al., 2017). Gwl also participates in the DNA damage checkpoint and/or response (DDR). The DNA damage checkpoint arrests cells in G2 in response to damaged DNA to protect them from genome instability (Zhou and Elledge, 2000). Studies in *Xenopus* egg extracts have revealed that the activation of DDR in these extracts, through the addition of double-stranded oligonucleotides, halts cell cycle progression by directly inhibiting Gwl (Peng et al., 2010). Moreover, removal of the double-stranded oligonucleotide results in mitotic entry, a response that is potentiated by Gwl overexpression and inhibited by Gwl depletion. Along the same line, removal of both Arpp19 and ENSA from mammalian cells delayed checkpoint recovery, suggesting that mitotic re-entry after DNA checkpoint recovery depends on the Gwl–Arpp19–ENSA–PP2A-B55 pathway (Wong et al., 2016).

Gwl and nutrient deprivation

In budding and fission yeast, the target of rapamycin complex 1 (TORC1) controls growth through its sensitivity to nutrients. In nutrient-rich media, TORC1 presents high activity; conversely, low-nutrient conditions promote TORC1 inactivation and quiescent G1 arrest. Interestingly, TORC1 negatively regulates Rim15 and Ppk18, the orthologues of Gwl in budding and fission yeast, respectively (Chica et al., 2016; Moreno-Torres et al., 2015). In budding yeast, under nutrient-rich media, the PKA-dependent phosphorylation of Rim15 maintains this protein in the cytoplasm owing to its association with 14-3-3 proteins (see poster), as previously described in *Drosophila*, and inhibits its kinase activity *in vitro* (Reinders et al., 1998). A decrease in carbon or nitrogen availability results in TORC1 and PKA inhibition, and thereby, in turn, activation of Rim15, which promotes translocation of Rim15 to the nucleus where it mediates G1 arrest (Wanke et al., 2005) (see poster). In accordance with an inhibitory role of PKA on Rim15-mediated G1 arrest, mutants yielding either elevated PKA activity or deletion of Rim15 result in a decreased expression of early meiotic genes and a perturbed G1 arrest in low-nutrient media (Reinders et al., 1998). Upon Rim15-dependent phosphorylation, the endosulfines Igo1 and Igo2 bind to PP2A-B55 in a cell cycle-regulated manner (Juanes et al., 2013). In low-nutrient media conditions, Rim15 is activated and phosphorylates Igo1 and Igo2, which results in the inhibition of PP2A-B55 (see poster). This inhibition will maintain mitogen-activated protein kinase 1 (Mkp-1)-dependent phosphorylation of the protein Sic1 at T173 and the stabilization of this protein, resulting in a G1 arrest through the inhibition of Cdk-Clb5,6 kinase activities (Moreno-Torres et al., 2015). Furthermore, this pathway also regulates the phosphorylation of the G1-specific transcriptional repressor Whi5. In its dephosphorylated form, Whi5 associates with and inhibits SCB-binding factor (SBF), a transcription factor that determines cell size required for G1/S transition. In low-nutrition medium or at early G1, high Rim15 activity preserves Whi5 phosphorylation and promotes G1/S transition at a smaller cell size. Consequently, the removal of Rim15 or Igo1 protein from yeast in poor medium conditions promotes high PP2A-B55 activity and dephosphorylation of Whi5, leading to an increased cell size at the G1/S transition (see poster) (Talarek et al., 2017). In fission yeast, the Ppk18–PP2A-B55 pathway also controls G1 arrest through the TORC2-dependent phosphorylation on S546 of the serine/threonine-protein kinase Gad8, the mammalian orthologue of Akt. In low-nutrient medium, Ppk18 prevents activation of PP2A-BB55, thereby maintaining the phosphorylation of Gad8 in its active form and arresting cells in G1 (Martín et al., 2017).

Conclusions and perspectives

In this Cell Science at a Glance article and accompanying poster, we have given an overview of the impact of Gwl in mitosis through the Arpp19–ENSA–PP2A-B55 pathway. We further described Gwl localization, the regulation of its activity, and the roles of Gwl during nutrient deprivation and oncogenic transformation of cells. However, new essential roles of this kinase in other unexpected cellular contexts are emerging. Such functions are mediated in some cases by the Arpp19–ENSA–PP2A-B55 signalling axis; however, the Gwl response also appears to involve other unknown cascades. The identification of additional Gwl targets is therefore essential to characterize these new transducing pathways; still, despite great efforts of several laboratories, no other Gwl substrates have been discovered yet. Hopefully, the future identification of these substrates will provide us with a more general picture of the cellular regulatory mechanisms and physiological functions controlled by Gwl kinase.

Competing interests

The authors declare no competing or financial interests.

Funding

The work of our laboratories is supported by the Agence Nationale de la Recherche (ANR-17-CE13-0011-02), by the Labex EpiGenMed (ANR-10-LABX-12-01), by a Fondation de France grant and by the Fondation pour la Recherche Médicale (FRM) (DEQ20150331692) to A.C.

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A high-resolution version of the poster and individual poster panels are available for downloading at http://jcs.biologists.org/lookup/doi/10.1242/jcs.222364.supplemental

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